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The purpose of this study was to improve the production of rabbit anti-atropine antisera for future analytical studies. There were two phases to this project: evaluation of methods to enhance atropine antisera titers, and antisera production and characterization. Anti-atropine antisera were prepared using a high-density conjugate. The radioimmunoassay performance characteristics were similar for both the Battelle and Walter Reed Army Institute of Research antisera. However, a lower detection limit and a more dynamic response range were obtained with the Battelle antisera. Minor changes were made in the atropine radioimmunoassay to improve its reliability. There were approximately 250 mL of atropine antisera collected during this project, providing a useful antisera supply adequate for 250,000 tests.

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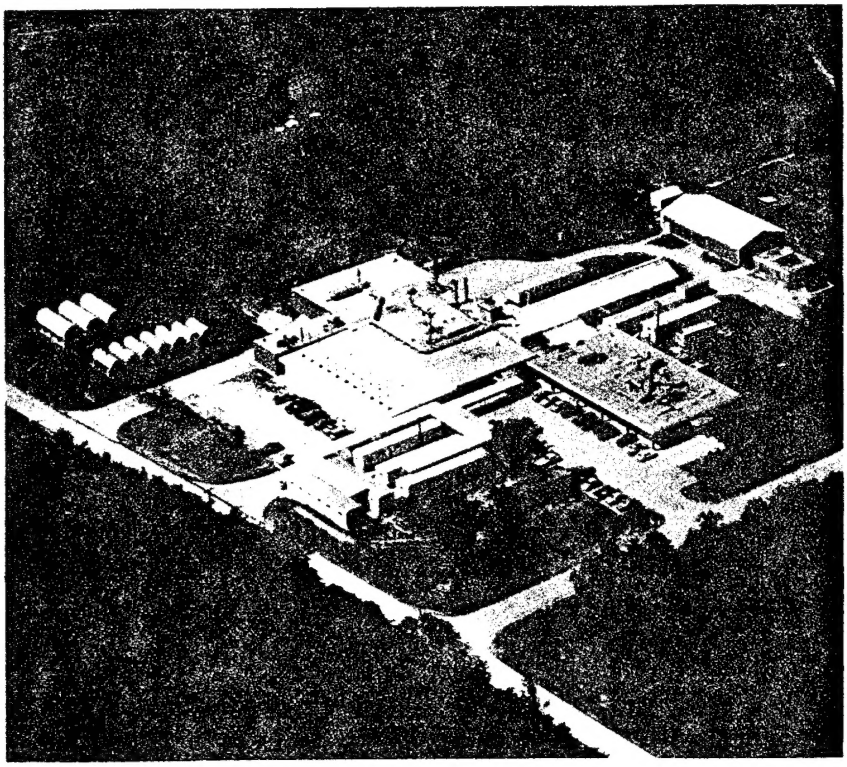

Task 91-27: Development of Procedures to Enhance Atropine Antisera Production in Rabbits

To

U.S. Army Medical Research and Development Command

July, 1994

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FINAL REPORT

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A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program

on

TASK 91-27: DEVELOPMENT OF PROCEDURES TO ENHANCE ATROPINE ANTISERA PRODUCTION IN RABBITS

to

U.S. ARMY MEDICAL RESEARCH AND
DEVELOPMENT COMMAND

July 1994

by

Dr. Larry Miller, Dr. Balwant Bhullar
Dr. Herman Benecke and Dr. Carl Olson

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health (NIH), Publication No. 86-23, Revised 1985).

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EXECUTIVE SUMMARY

The purpose of this study was to improve the production of rabbit anti-atropine antisera for future analytical studies. For Phase I of this project, a high-density atropine-protein conjugate was prepared and characterized. This atropine-PABGA-thyroglobulin conjugate had an atropine:protein molar ratio of approximately 419:1. Atropinase-free or untested New Zealand White rabbits were injected periodically with this new high-density conjugate, or with the previously prepared atropine-PABA-BSA conjugate, in Freund's or TITERMAX™ adjuvant over 284 days. Neostigmine, an esterase inhibitor, was incorporated with the immunogen and adjuvant in another group of rabbits in order to prevent degradation of the atropine conjugate. Antisera from these five immunization groups were evaluated initially using a binding assay. Of the ten animals in each group, there were more high responders (titer ≥ 1:800) in the atropine-PABGA-TG group with Freund's adjuvant and neostigmine than in the other groups. The rabbits immunized using the TITERMAX™ adjuvant did not produce any antibodies, presumably due to the severe reactions obtained with this adjuvant. However, the mean titers of those groups of rabbits responding were not statistically different. Significant factors were number of injections, type of adjuvant, and immunogenetics of the responder rabbits.

In Phase II, antisera from five rabbits having the highest titer were characterized further in the atropine RIA. The titers of these sera were optimized and were equivalent (1:1000-1:4000) to the WRAIR antisera (1:1600). At the optimal dilution, standard curves with a detection limit of 12.5 pg/50 μL (0.25 ng/mL) and a response range extending to 3200 pg/50 μL (64 ng/mL) were generated, resulting in a sixfold improvement over the WRAIR antisera in the method sensitivity. The standard curve slope, y-intercept, correlation coefficient, and R/T values were comparable to those obtained using the WRAIR antisera. For specificity studies, significant crossreactivity was obtained with l-hyoscyamine (13-50 percent) and d,l-homatropine (5-50 percent). The WRAIR anti-atropine antisera also crossreact but to a lesser extent with l-hyoscyamine (2.4 percent) and d,l-homatropine (3.3 percent). Scopolamine, acetylcholine iodide, atropine methyl nitrate, tropic acid, and tropine showed no significant crossreactivity (<3 percent).

Using Scatchard plots, the average binding constants for the Battelle antisera (0.05 to 0.14 nM^{-1}) were twofold to fivefold higher than those for the WRAIR antisera (0.28 nM^{-1}).

In conclusion, additional anti-atropine antisera were prepared using a high-density conjugate. The RIA performance characteristics were similar for both the Battelle and the WRAIR antisera. However, a lower detection limit and a more dynamic response range were obtained with the Battelle antisera. Minor changes were made in the atropine RIA to improve its reliability.

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DEVELOPMENT OF PROCEDURES TO ENHANCE ATROPINE ANTISERA PRODUCTION IN RABBITS

1.0 INTRODUCTION

A primary mission of the U.S. Army Medical Chemical Defense Program has been to develop improved treatment regimens for nerve agent poisoning. Determination of the pharmacokinetics of an improved treatment regimen's components (atropine and oxime) is a critical portion of the submission to the U.S. Food and Drug Administration for approval to field the improved treatment regimen. The currently accepted method for measuring atropine levels in blood (database from which atropine pharmacokinetic parameters are generated) is a radioimmunoassay (RIA) method which requires atropine antisera of acceptable titer. To support anticipated projects, additional atropine antisera must be generated. However, previous attempts to generate atropine antisera using information from published reports and from communications with researchers were significantly below the target antisera titer (1:1600). The purpose of this task was to investigate several approaches for enhancing atropine antisera formation in rabbits.

A radioimmunoassay (RIA) for atropine was developed by Wurzberger et al.[1] This method has been used by the U.S. Army at the Walter Reed Army Institute of Research (WRAIR) to determine atropine concentrations in samples before being transferred to Battelle Columbus Laboratories.[2] At Battelle Columbus Laboratories the method was automated to improve the accuracy and speed of sample analysis. Task 91-27 was conducted to produce additional, high-quality anti-atropine antisera for use in future analyses.

2.0 MATERIALS AND METHODS

The purpose of this task was to evaluate alternative approaches for the production of adequate titers of atropine antisera in rabbits. The project was conducted in two phases according to MREF Protocol 83 (Appendix A), incorporating the atropine RIA (Battelle Standard Operating Procedure [SOP] TOX. VI-014, Appendix B) and other SOPs from Task 89-02. In Phase I, three different atropine-protein conjugates were prepared. In Phase II, rabbit

antisera were prepared using three approaches in an effort to produce high titer antisera having characteristics similar to those of the WRAIR antisera.

2.1 Phase I. Preparation and Analysis of Atropine-Protein Conjugates

2.1.1 Preparation of Atropine-Protein Conjugates

Various atropine-protein conjugates were prepared as immunogens using the procedure described by Wurzberger et al.[1] For the diazotization reaction, 137 mg (1 mmol) of p-aminobenzoic acid (PABA) or 266 mg of N'-p-aminobenzoyl glutamic acid (PABGA) from Sigma Chemical Co., St. Louis, MO, was dissolved in 6 mL ice-cold 0.2N HCl by stirring in an ice-water bath. Next, 180 mg (1.3 mmol) of $NaNO_2$ (Sigma) dissolved in 2.0 mL ice-cold, deionized water was added dropwise with stirring. After adding 4 mL of ice-cold, 50 percent dimethylformamide (DMF; Sigma), the reaction was allowed to proceed for 50 min at 0° to 2°C. The solution changed from clear to orange-brown due to the formation of azo groups from the diazotization of PABA or PABGA. To quench the unused $NaNO_2$, 29 mg (0.3 mmol) of sulfamic acid (Sigma) was dissolved in 1 mL ice-cold deionized water and added at the conclusion of the incubation.

The activated PABA and PABGA crosslinkers were coupled then to atropine sulfate. For this reaction, 778 mg (1.33 mmol) of atropine sulfate (Sigma) was dissolved in 20 mL 0.1M sodium borate, pH 9.0, while stirring on ice. The diazotized PABA or PABGA was added dropwise, with stirring, to the atropine sulfate solution. The pH was maintained at 9.0 with 1N NaOH for 30 min and then the reactants were stirred overnight at 0° to 2°C in the dark. The diazo reaction mixture was brought to room temperature. At this stage, the reaction mixture was dark red. Next, 18.6 mL of buffer (0.2M KH_2PO_4, 0.1 percent NaN_3, pH 6.5) was added, resulting in a final pH of 7.5. The total volume of the reaction mixture was approximately 50 mL.

Finally, the atropine-crosslinker (PABA or PABGA) was coupled to a carrier protein. First, 108.5 mg of N-hydroxysulfosuccinimide (sulfo-NHS; Pierce, Rockford, IL) and 1.92 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Pierce) were added simultaneously to the atropine-PABA or atropine PABGA solutions, resulting in final concentrations of 10 mM and 200 mM, respectively. Next, 200 mg (0.298 μmol) of thyroglobin (TG) or bovine

serum albumin (2.98 μmol; BSA) from Sigma Chemical Co. was dissolved in 30 mL or 20 mL, respectively, of 1M KH_2PO_4, 0.1 percent NaN_3, pH 8.5. The protein solutions were added to atropine-PABA or atropine-PABGA and the pH adjusted to 8.5 using 2M K_2HPO_4. The solution was stirred for 1 hr at room temperature, and then for 16 to 24 hr at 2° to 5°C. The conjugates resulting from this coupling reaction had an orange appearance.

Any free atropine was removed from the atropine-crosslinker-protein conjugates by dialysis. The solutions were transferred to preboiled dialysis tubing (12,000 MW cutoff) and dialyzed for 6 hr against 2L of 0.15M NaCl at room temperature. The conjugate was further dialyzed at 2° to 8°C against 2L of 0.15M NaCl in a stirred container for 10 hr, and dialysis was repeated under similar conditions for 16 hr, and suspended material, if any, was removed from soluble atropine-protein conjugate in the solution by centrifugation at 8,000 X g for 1 hr at room temperature. The protein concentration of the conjugate solution was adjusted to 1 mg atropine-protein/mL using 0.15M NaCl as diluent. The conjugate solution was aliquoted into polyethylene cryovials and stored at -25° to -15°C.

2.1.2 Analysis of Conjugates by UV Spectroscopy

Absorption spectra of the atropine-protein conjugates were determined using a Beckman DU-50 spectrophotometer and 1-cm wide quartz cuvettes. Absorbency at 280 nm was determined to estimate protein content by using an absorptivity value of 0.66 for a 1 mg/mL protein solution of BSA and 1.34 for a 1 mg/ml solution of TG.[3] Molar amounts of protein were calculated using a molecular size of 69,000 for BSA[4] and 669,000 for TG.[5] Absorbance at 350 nm was used to quantitate the azo groups of the atropine-protein conjugates, and the molar concentration was calculated using a molar extinction coefficient of 22,000.[6] Molar ratios of atropine:protein were calculated for each conjugate.

2.1.3 Analysis of Atropine-Protein Conjugates by ELISA

Conjugates were evaluated for immunoreactive atropine using an inhibition ELISA method as described by Voller et al.[7] To coat the microwell plates (Immulon IV, Dynatech, Chantilly, VA), atropine-PABGA-TG diluted to

20 ng/mL in coating buffer (0.2 M $NaHCO_3$, 0.1 percent NaN_3, pH 9.2) was added to each microwell. The plate was then covered with Parafilm™ and left overnight at room temperature. Diluted rabbit antisera (1:5000 in assay buffer containing 50 mM Tris-HCl, 500 mM NaCl, 0.1 percent Tween 20, 1 percent BSA, and 0.1 percent NaN_3, pH 8.0) were mixed with equal volumes of atropine standards from 0.002-40 ng/mL in assay buffer and incubated 18-24 hr at 2° to 5°C. The conjugate-coated microwells were washed four times with wash buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1 percent Tween 20, 1 percent BSA, 0.1 percent NaN_3, pH 8.0) and any remaining wash buffer was removed by gently tapping inverted plates on a paper towel. Three 200-μL samples of one of the antisera-atropine solutions were added to microwells and sealed with Parafilm™. After agitating on an orbital shaker (VWR) at a setting of 150 for 3 hr at room temperature, the plates were washed four times with wash buffer. A second antibody (goat anti-rabbit IgG, alkaline phosphatase from Sigma Chemical Co.) was then diluted 1:500 in assay buffer and 200 μL was added to each microwell. The plates were resealed with Parafilm™ and agitated on the orbital shaker for 3 hr at room temperature. The excess antibody was removed by washing microwells four times with wash buffer. Nitrophenylphosphate (Sigma Chemical Co.), a chromogenic substrate, was diluted to 1 mg/mL in 1M diethanolamine buffer at pH 10.0 and 200 μL quantities were added to each well. After shaking the plate on an orbital shaker for 30 min at room temperature, the absorbance at 405 nm was measured with a Molecular Devices Plate Reader interfaced with an 80386 Intel microprocessor computer. SOFTmax™ software (Molecular Devices, Menlo Park, CA) was used for data analysis to calculate mean absorbance, standard deviation, and percent coefficient of variation, and for fitting curves of absorbance vs. concentration of atropine-standard.

2.1.4 Enzyme Degradation of Conjugates

Atropine-protein conjugates also were treated with rabbit serum esterase and rabbit liver esterase (Sigma Chemical Co.). For these reactions, 2 mL of 90 μM atropine equivalents for the conjugates or 90 μM atropine was incubated with 2 mL of undiluted rabbit serum (Sigma Chemical Co.,) or 2 mL of 1 mg/mL rabbit liver esterase in 50 mM K_2HPO_4, pH 7.0 for 18 hr at 25°C. Reactions were stopped by adding trichloroacetic acid to a final concentration

of 10 percent to inactivate the enzymes, and then preparations were centrifuged at 10,000 X g for 10 min at 4°C. The supernatant was collected and analyzed using the atropine RIA. Atropine degradation was monitored also by measuring tropic acid production using HPLC.[8] This method was performed using a C_{18} column (Altex) on a Beckman Model 322 System with a 114 Pump and Waters Model 712 WISP Autoinjector and a flow rate of 1 mL min^{-1} for a 17 percent aqueous acetonitrile-0.3 percent phosphoric acid mobile phase. The absorbance at 204 nm was measured with a Beckman Model 163 Wavelength Detector.

2.2 Phase II. Antisera Production and Characterization

2.2.1 Antisera Preparation

Anti-atropine antibodies were produced in female New Zealand White rabbits (Hazleton Research Animals, Denver, PA) weighing 1.5 to 3.0 kg. The blood of rabbits was screened for atropinase activity using MREF SOP-88-48 prior to beginning the immunization schedule. Animal care and antisera production methods are described in MREF Protocol 83 and its amendments (Appendix A). Rabbits were immunized with different atropine-protein conjugates in Freund's adjuvant (Sigma Chemical Co.), TITERMAX™ (CytRx Corp., Norcross, GA), or Freund's adjuvant containing 0.2 mM neostigmine methyl sulfate (an esterase inhibitor; Sigma Chemical Co.,) every 3 weeks for 6 months. For immunizations using Freund's adjuvant with or without neostigmine, 1 mg of atropine-protein conjugate in 1.5 mL phosphate buffered saline (PBS) was mixed with an equal volume of adjuvant in an 18 mL syringe. Using an OMNI 2000 homogenizer (TekMar, Cincinnati, OH) for six 10-sec treatments, an emulsion was formed and was stored in a refrigerator overnight. Freund's complete adjuvant was used only for the initial immunization; Freund's incomplete adjuvant was used thereafter. Each rabbit received 1.0-1.5 mL of antigen-adjuvant emulsion given intramuscularly in both hind legs and subcutaneously at 10-15 sites in the sub-scapular region. For TITERMAX™, 1 mg of atropine-protein conjugate in a 0.4 mL volume for the initial injection, and in a 0.2 mL volume for all subsequent preparations, was mixed with an equal volume of the adjuvant by using the OMNI 2000 homogenizer, and the emulsion was injected at four subcutaneous sites. Animals were clipped of hair at the

injection sites prior to each injection according to MREF Method No. 1/General. Blood samples were obtained from the middle ear artery on Day 0 and approximately 10 days after the 3rd, 5th, 7th, and 9th injections. The blood was allowed to clot at room temperature and then stored overnight in a refrigerator. The serum was separated by centrifugation at 600 X g for 20 min at room temperature, collected, and stored at -25° to -15°C.

2.2.2 Assay Automation

The automated atropine RIA was used for antisera characterization studies (see Atropine RIA SOP in Appendix B). The addition of assay samples and reagents to RIA tubes (standards, samples, buffer, normal serum, anti-atropine antibody, and ^{3}H-atropine) was performed using a Tecan RSP Model 5052 Robotic Sample Processor (Tecan U.S., Hillsborough, NC). The system was controlled using an IBM Model 55SX computer and the Application Method Integrator (AMI) Software (v. 7.31; Tecan U.S.). Addition of 50 percent saturated ammonium sulfate solution, distilled water, and Hydrofluor (National Diagnostics Inc., Manville, NJ) was performed manually. Samples were counted in a Beckman LSC Model 6000 using the settings for tritium, and the data were analyzed using RIACalc™ software, Version 2.65 (Pharmacia LKB Inc., Gaithersburg, MD) and a 80486 Intel microprocessor computer.

2.2.3 Antisera Atropine-Binding Activity

Anti-atropine antibody binding activity was monitored by determining the ratio of percent bound cpm/total cpm (%B/T) for sera at a 1:100 dilution. The atropine RIA was performed according to the revised SOP (February 21, 1994; Appendix B). Addition of diluted antisera, radiolabeled atropine, and other reagents to RIA tubes was performed with a RSP 5052 Robotic Sample Processor (Tecan U.S.). PBS was used in place of unlabeled atropine in the reaction.

2.2.4 Antisera Characterization

Antisera titration curves were prepared using the atropine binding activity assay data. Rabbit anti-atropine sera were diluted twofold in 1 percent heat-inactivated, normal rabbit serum in PBS and used in place of samples in the atropine RIA. The titer was defined as the nearest twofold dilution producing half-maximal binding (50 percent B_o/T). Statistical analyses of group titers were performed using Sigma Stat software, Version 1.01 (Jandel Scientific, Carle Madera, CA).

The antisera dilution was optimized for the atropine RIA. Three dilutions between the 50 percent to 100 percent maximal binding were selected from the titration curves. The optimal dilution was determined from the detection limit, response range, (B/T) max, (B_o/T), and slope of logit-log plots for atropine standard solutions at 0.25 to 64 ng/mL, or 12.5 to 3,200 pg/50 μL. These plots and parameters were obtained using the RIACalc™ software.

The specificity of individual anti-atropine antisera was evaluated by substituting compounds structurally related to atropine or its metabolites in the atropine RIA. L-hyoscyamine hydrochloride, atropine methyl nitrate, (-)scopolamine hydrochloride, d,l-homatropine hydrobromide, acetylcholine iodide, tropine, and d,l-tropic acid were purchased from Sigma Chemical Co. These compounds and atropine sulfate were prepared in 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.5 containing 1 percent heated inactivated normal rabbit serum to give final concentrations of 0.2, 2, 20 and 200 nM. The 50 percent inhibition concentration was determined from these RIA curves.

The antibody binding constant for atropine was determined using Scatchard plots of the inhibition curves. Independent determinations were made using data from two atropine standard curves, and the values were averaged. Scatchard plots were prepared using the RIACalc™ software by plotting B/F versus nM bound atropine.

3.0 RESULTS

3.1 Phase I. Preparation of Atropine-Protein Conjugates

3.1.1 Approach 1. Preparation of High-Density Atropine-Protein Conjugate

Background. An organic compound, such as atropine, does not elicit antibody formation, but can be rendered immunogenic by coupling it to a large polymer such as a protein.[9] Rabbits are often chosen for antisera production because of their strong response to hapten-protein conjugates.[10] Atropine can be conjugated with BSA (mol. wt. 69,000) at a level of approximately 35 residues of atropine per molecule of BSA. This BSA-atropine conjugate was used as the immunogen to form antibodies in rabbits. Although the antisera were usable in an RIA for atropine, the titers were low (1:20 to 1:100) relative to the WRAIR antisera (1:1600). Conversations with WRAIR scientists who obtained this 1:1600 antisera (Drs. Smallridge and Verma) revealed that only one in eight rabbits produced a high titer antiserum (≥1:1000).

A possible explanation for the production of low titer antisera is instability of the ester bond in the atropine moiety (Figure 1, Appendix C). Atropine contains this ester linkage between the tropine and tropic acid moieties, and it is susceptible to hydrolysis at a high pH[11] or due to various esterases in rabbits.[1] A significant improvement in immunogenicity may be achieved by using an esterase inhibitor, by increasing the number of atropine residues on the carrier protein, or by using different adjuvants.

Approach. The conjugation chemistry described previously and originally used by Wurzberger et al.[1] was modified to enhance atropine incorporation and to prevent atropine degradation. Buffers at pH 8.5 were used in place of 1 N sodium hydroxide, pH 14, to reduce high pH conditions that might create cleavage of the atropine molecule at the ester site. The coupling reaction performed at pH 8.5 also deprotonates more of the lysyl groups, allowing for greater atropine incorporation. Dimeric thyroglobulin, having 148 lysyl residues,[12] was used as a carrier protein since it has approximately twofold to threefold more available attachment sites than BSA with its 62 lysyl residues.[4] Therefore, it should be possible to incorporate 200-300 atropine molecules for each molecule of thyroglobulin. This higher level of coupled

atropine would theoretically improve antibody formation by providing more atropine residues. N'-p-Aminobenzoyl glutamic acid (PABGA) was also used as a crosslinker because it is a dicarboxylic acid, rather than a monocarboxylic acid like PABA, providing two sites for attachment to the protein. PABGA also provides a longer chain, an additional five carbon atoms, for crosslinking atropine to the protein. Theoretically, this makes the atropine more available for entering into an immune response and resulting in increased antibody formation.

Conjugation Reaction. In the first step of the coupling reaction, the amino functional group of PABGA or PABA was converted to a diazonium salt by reaction with a 50 percent molar excess of sodium nitrite under acidic conditions. The unused sodium nitrite was quenched by adding sulfamic acid.

The diazotized PABGA or PABA was incorporated into the phenolic ring of the tropic acid moiety by reactions with a 30 percent molar excess of atropine at an alkaline pH and low temperature. It was important to utilize all of the diazotized crosslinkers in this reaction so that they would not be free to react with benzyl groups of the protein, resulting in side reactions that would crosslink the protein molecules and create insoluble aggregates to mask the lysyl groups needed for atropine incorporation. Atropine sulfate was reacted with the diazotized PABGA or PABA solution under alkaline conditions at 0° to 2°C for 16 to 68 hr in the dark. During this reaction, a buffered solution was used to reduce the need for pH adjustment with 1N sodium hydroxide (pH ~14) to minimize localized pH extremes which would result in hydrolysis of the atropine.

The atropine-PABGA or atropine-PABA conjugates were coupled to the free amino groups on the carrier proteins, TG or BSA, using carbodiimide chemistry. During this reaction, the lysyl amino groups on the protein form stable amide linkages with the activated carboxyl moieties of atropine-PABGA (dicarboxylic) or atropine-PABA (monocarboxylic). There are 148 lysyl residues in TG and 62 in BSA, resulting in 44.2 μmol of lysyl groups for 200 mg TG and 179 μmol lysyl groups for 200 mg BSA. Assuming the diazotized PABA or PABGA is at 1 mmol, there is a 22-fold and a 5-fold molar excess of the diazotized PABA or PABGA relative to the lysyl groups of TG or BSA, respectively. Although the optimal pH for carbodiimide conjugation is 5-6,[13] a higher pH (8.5) was used since the pK_a of the lysyl ϵ-amino group is 10.53.[14] The lysyl ϵ-amino group

is reactive only in the unprotonated state; therefore, an alkaline pH would favor this reaction. In addition, carbodiimide is more stable in neutral to slightly alkaline pH, resulting in greater reaction efficiency.

Any unbound atropine and crosslinking reactants were removed from the conjugated protein by dialysis against 0.15 M sodium chloride in a stirred container. The conjugate solution had an orange appearance, resulting from the azo linkages of this coupling chemistry. The atropine-protein conjugate chemistries are shown in Figure 2 (Appendix C).

UV Absorption Spectroscopy. UV spectral analysis of the conjugates was performed to determine the extent of incorporation of atropine into TG or BSA. UV absorption spectra of atropine-protein conjugates are shown in Figure 3 (Appendix C) and data are summarized in Table 1 (Appendix D). Atropine has characteristic maximum wavelength (λ_{max}) absorption peaks at 250 nm, 256 nm, and 262 nm that are distinct from the λ_{max} of tyrosyl residues of the protein (278 nm). The atropine-protein conjugate has a broad absorption peak at 268 nm, corresponding to the tyrosyl, atropine, and crosslink compound (PABA or PABGA) moieties. A second absorption peak occurs at 350 nm, corresponding to the azo groups of the crosslinker.

Using the appropriate absorptivity values, the molar amounts of the azo groups and protein were calculated, and the molar ratios of atropine:protein were determined. The highest molar ratio was obtained with atropine-PABGA-TG (419:1). The TG conjugates had higher atropine incorporation than BSA conjugates due to the higher number of lysyl groups (296 for TG and 62 for BSA). In addition, the PABGA also produced 1.3-fold to 3.5-fold higher atropine incorporation than PABA. The two-fold higher number of carboxyl groups on the PAGBA and the higher number of lysyl groups in TG resulted in greater atropine coupling. These results only indirectly indicate the covalent coupling of atropine onto TG or BSA through the PABGA or PABA since only the azo linkage is being quantitated and not the atropine. The broad absorption peak at 278 nm also indicated the incorporation of atropine, but quantitation was not possible due to the absorption peak overlap between atropine, PABA, PABGA, and tyrosine.

ELISA Analysis. The amount of atropine coupled to protein was determined by an inhibition enzyme-linked immunosorbent assay (ELISA). Rabbit antisera obtained previously were used for these studies. These antisera contained antibodies to atropine-PABA, and BSA. To prevent reactivity with the PABA and the BSA carrier protein, microwell plates were coated with conjugate having a different crosslink compound (PABGA) and carrier protein (TG). For this combination (anti-atropine-PABA-BSA antibodies and atropine-PABGA-TG antigen), only the anti-atropine antibodies would be detected.

Rabbit anti-atropine-PABA-BSA sera at a final dilution of 1:10,000 and varying concentrations of atropine (0.002 to 40 ng/mL) were incubated in these coated plates. During this incubation (overnight at 4°C), antibodies were complexed either with free atropine or the solid-phase atropine of the atropine-PABGA-TG conjugate. The solid-phase atropine-PABGA-TG was reacted with a labeling antibody (a goat anti-rabbit IgG, alkaline phosphatase conjugate). After washing excess reagents from the microwell, a chromogenic substrate (p-nitrophenyl phosphate) was added, producing a colored product with an intensity inversely proportional to the amount of atropine in the solution. An inhibition curve having an ID_{50} of 0.6 ng/mL was obtained (Figure 4, Appendix C) indicating the presence of atropine in the atropine-PABGA-TG conjugate. TG-coated wells served as a negative control.

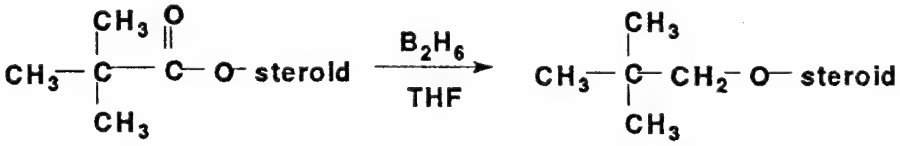
An additional study also was performed to determine if tropic acid was produced from atropine hydrolysis during the conjugation reactions. Antisera to atropine-PABA-BSA would contain antibodies to tropic acid if there had been significant hydrolysis of atropine during the coupling reaction. These antibodies would react with tropic acid sites on the atropine-PABGA-TG conjugate, if they were present, and would be inhibited. The inhibition ELISA was repeated using tropic acid (0.002^{-64} ng/mL). At the highest concentration, there was a 17 percent inhibition in binding. This result indicates that most of the atropine was intact and only a small fraction was present as tropic acid.

3.1.2 Approach 2. Atropine Analogue Synthesis and Conjugation

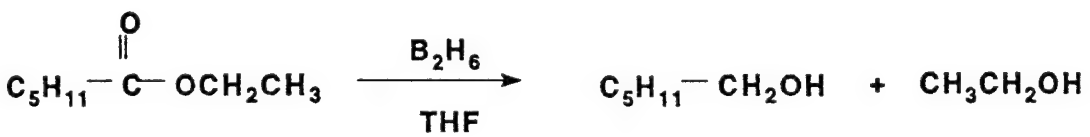
Background. A second approach to synthesize an atropine analogue which did not possess the hydrolyzable ester present in atropine was evaluated. After consideration of potential chemical transformations, it was decided that conversion of the ester of atropine to an ether was feasible and the resulting analogue should be resistant to hydrolysis. Potentially, conjugation of this analogue to a protein would lead to enhanced atropine antisera formation in rabbits due to the anticipated stability of the bound hapten.

Approach. Two different approaches were investigated for preparation of the desired atropine ether analogue. The first approach involved a single-step conversion of atropine to the ether derivative. A multiple-step synthetic approach to formation of the ether analogue was also evaluated. Following are descriptions of the two approaches investigated and the results that were obtained.

Direct Reduction of the Ester Function. Diborane in tetrahydrofuran (THF) has been reported to convert sterically hindered esters, such as the steroidal (lanostanyl) ester of pivalic acid, to the corresponding neopentyl ester,[15] as shown below.



On the other hand, the reaction of unhindered esters (such as ethyl caproate) with diborane in THF gives predominantly the alcoholic cleavage products as shown below.



It was postulated that the total steric hindrance about the carbonyl region and the O-alkyl region of the ester function in atropine was similar to the steric hindrance about the lanostanyl pivalate ester function shown above. The desired reduction of atropine by diborane in THF to its ether derivative is shown in Figure 5 (Appendix C). However, when atropine was reacted with diborane in THF (purchased or generated in situ from sodium borohydride and boron trifluoride) under a variety of conditions, the desired reduction did not occur, and undesirable side reactions did occur. The diborane in THF was found to react minimally with atropine at ambient temperatures, even after extensive time periods. Atropine was shown to undergo N-methyl group cleavage when refluxed with internally generated diborane. To prevent this demethylation reaction, a removable benzyl quaternary ammonium salt of atropine was prepared and characterized. However, the reaction of this atropine derivative with in-situ generated diborane led to quantitative cleavage of the ester group at reflux or ambient temperatures. These results suggest that the quaternary nitrogen atom in this atropine derivative may enhance the reactivity of the borohydride ion toward cleavage of the ester group since a similar cleavage was not noted in the reaction of atropine with the same reagents at ambient temperatures. One reaction, which was not tried, was that of the benzyl quaternary salt of atropine with purchased borane.

Multiple-Step Route to Atropine Ether Derivative. A potential multiple-step pathway to prepare the desired atropine ether derivative (A) is shown in Figure 6 (Appendix C). The first step in this synthesis involved blocking of the tertiary nitrogen atom of tropine with a removable benzyl group to generate the quaternary ammonium intermediate (B). The removable benzyl group prevented unwanted substitution at this site in the next reaction. Intermediate B was successfully prepared and characterized. The next reaction involved the reaction of a chloro/benzyl ether derivative of tropic acid (C). However, the overall synthesis did not progress past this step due to difficulty in preparing the intermediate C.

Two potential routes to prepare intermediate C are described below and presented in Figures 7 and 8 (Appendix C). The reaction of intermediates B and C would involve the displacement of the primary chloro group of intermediate C by the secondary hydroxyl group of intermediate B. If the nitrogen atom in B were not blocked by the benzyl group, reaction with C would occur nearly

exclusively at that site. The alcohol function of the tropic acid derivative C will also be blocked to prevent the intramolecular reaction of this function with the chloro group also present in intermediate C. Intermediate D will have the desired structural features of the atropine ether derivative (A) and will be converted to this compound by removal of both blocking benzyl groups by simple hydrogenolysis as shown in the last reaction.

The first synthetic route to intermediate C, which was investigated in this study, is shown in Figure 7 (Appendix C). The first step in this route involves the preparation and characterization of the ethyl ester of tropic acid (intermediate E), which was accomplished. However, the conversion of intermediate E to intermediate F was not achieved by any of several approaches. The first attempt involved the initial reaction of E with butyl lithium at -78°C. The objective of this reaction was to deprotonate the hydroxyl function to generate the reactive alkoxide anion, which was to be alkylated by benzyl bromide to form the desired ether function. However, when this reaction mixture was allowed to warm to ambient temperature in the presence of benzyl bromide, only the elimination product F' was detected and isolated. This compound was conclusively identified by H-1 NMR spectroscopy. This compound apparently resulted from competitive formation of the benzylic carbanion, which was stabilized by the carbonyl group of the ester function. An apparent cause for this elimination was the ability of the intermediate carbanion to eliminate hydroxide ion and generate a double bond, which was conjugated both to the phenyl ring and to the ester carbonyl group.

To avoid generation of this reactive anion, the reaction was altered by use of silver oxide instead of butyl lithium. It was expected that the silver oxide would initially abstract a bromide ion from benzyl bromide to form the stabilized benzyl carbonium ion, which was expected to react at the alcohol site of intermediate E to give the desired benzyl ether derivative. However, the only product isolated from this reaction was the undesired intermediate F'. Apparently, the oxide ion of silver oxide was basic enough to react with E and generate the same reactive carbanion, which again underwent the elimination reaction. Accordingly, the reaction was modified again by using the relatively non-basic sodium carbonate and silver nitrate to generate the reactive benzyl carbonium ion. Sodium carbonate was used to react with the nitric acid. In this modification, only a trace of the desired product was detected by mass

spectroscopy. However, the undesirable elimination reaction was avoided by this final modification.

Due to the problems described in preparing intermediate C, the alternate approach shown in Figure 8 (Appendix C) was initiated to prepare this compound. The first step in this route involves the reduction of diethyl malonate to 2-phenyl-1,3-propanediol (H). This intermediate has been prepared and characterized. However, the effort was stopped at this stage after reviewing the status of the approach and evaluating the probability of successfully preparing such an atropine derivative. The next step envisioned involved the selective chlorination of one of the hydroxyl groups in intermediate H to form intermediate I. The final step would have involved alkylation of the hydroxyl group in I with benzyl bromide under phase transfer conditions (PTC). The corresponding elimination problem which was observed in the attempted alkylation of intermediate E should not occur in the conversion of I to C since the undesired benzylic carbanion would not possess the extra stabilization afforded by an ester carbonyl group. Precedents were found in the literature which indicated that the desired alkylation had been performed on substrates which had structural features similar to those of intermediate I.

3.1.3 Approach 3. Esterase Inhibitor with Atropine-Protein Conjugate.

Atropine is an ester of tropic acid and tropine. This ester is susceptible not only to chemical cleavage but also to enzymatic hydrolysis. Rabbits and other species contain cholinesterases and other esterases in blood and cells which can degrade atropine.[1] Antibody formation to atropine requires the processing and presentation of the atropine-protein conjugate in a manner that elicits an immune response. As part of the immunization process, macrophages process the antigen and present fragments to antigen-reactive T-helper lymphocytes.[16] As part of the antigenic processing, the antigen (atropine-protein conjugate) undergoes endocytosis and is contained in endosomes in the macrophage intracellular environment. The endosomes are at low pH (4-5), and fuse with another subcellular organelle, lysosomes, which contain various enzymes including esterases.[17] The esterases may degrade a substantial amount of the atropine molecules attached to the carrier protein,

leaving only the tropic acid attached. The addition of an esterase inhibitor might prevent this degradation.

Neostigmine is an esterase inhibitor that is active in biological systems at 0.2 mM concentration[18,19] and has a chemical structure similar to atropine. Neostigmine at a 0.2 mM final concentration was mixed with 1 mg of atropine-protein conjugate in an adjuvant and injected into rabbits. Assuming 100 molecules of atropine are incorporated into each thyroglobulin protein molecule, this ratio is equivalent to a 1400-fold molar excess of neostigmine to atropine-protein conjugate. As the adjuvant-antigen mixture undergoes endocytosis by macrophages and other antigen-presenting cells, it was anticipated that the neostigmine would react with esterases, preventing enzymatic degradation of atropine coupled to the protein, but still allowing the atropine-protein conjugate to be processed and presented by the accessory cell to T-helper lymphocytes and B-lymphocytes, resulting in antibody formation. Esterase inhibitors such as diisopropylfluorophosphate and sodium fluoride were not considered for use in this study because of their toxicity. Other esterase inhibitors, such as diphenylcarbamyl fluoride, ebelactone, forphenicine, bestain, amastatin, arphamenine, and leupeptin, were not considered because their chemical structures are unrelated to atropine.

3.2. Phase II. Preparation and Characterization of Anti-Atropine Antisera

3.2.1 Optimization of Atropine RIA

The atropine RIA was used for the purpose of characterizing the antisera from this project. Variable results were obtained with this method in regard to the acceptability of the standard curve. Improvements to correct this variability were defined and reviewed with the U.S. Army Contracting Officer's Representative (COR) prior to these studies. The factors studied were H^3-atropine levels, atropine standards preparation, amount of normal rabbit serum (NRS), antibody dilution, ammonium sulfate precipitation, and liquid scintillation counting time. From these studies, optimal conditions for the atropine RIA were determined.

Experiments were conducted using conditions outlined in Table 2 (Appendix D). The amount of tracer was reduced to 4,000 cpm to allow for

acceptable counting statistics while using only a minimum amount of H^3-atropine to improve assay sensitivity by selecting for high affinity antibodies. The amount of normal rabbit serum was increased to 150 µL to improve ammonium sulfate precipitation of the atropine-antibody complex, resulting in improved precision. The antibody dilution was increased to 1:2000 to reduce the amount of low affinity binding proteins and antibody, allowing only the high affinity antibodies to bind atropine and result in a lower detection limit of 12.5 pg/50 µL. The atropine standards were prepared at a constant volume of 50 µL, using twofold dilutions instead of varying volumes from 10 to 50 µL, resulting in improved precision and ease of preparation. The incubation time and temperature for the RIA was optimal at 1 hr at room temperature followed by the addition of ammonium sulfate and overnight incubation at 4°C. The 1 hr at room temperature was adequate time for the atropine-antibody reaction to reach equilibrium, and the overnight ammonium sulfate precipitation resulted in more efficient precipitation of the atropine-antibody complexes. Lastly, the increased counting time or preset 2 percent CV allowed for improved method precision by optimizing the counting statistics. A representative RIA inhibition curve is shown in Figure 9 (Appendix C). The optimal conditions described above were reviewed with the U.S. Army COR and approved for subsequent studies using the RIA method.

3.2.2. Antisera Production and Characterization

Antisera Production. Rabbits were used for the production of anti-atropine antibodies. Based on previous information and experience, the rabbits were immunized with 1 mg atropine-thyroglobulin conjugate, with or without neostigmine, mixed in adjuvant. Previously, Freund's and RIBI adjuvants had been used and produced equivalent results. In this study, Freund's adjuvant and Hunter's TITERMAX™, which was reported to be superior to Freund's adjuvant (manufacturer's brochure), were compared. Atropine-PABGA-TG, a high-density conjugate, was chosen for three of the immunization groups (Table 3, Appendix D) in combination with Freund's adjuvant only (Group 1), TITERMAX™ adjuvant (Group 2), or Freund's adjuvant with neostigmine (Group 3). An additional group of three rabbits were immunized with a lower-density atropine conjugate, atropine-PABA-BSA, in Freund's adjuvant (Group 4). The

three rabbits were from a previous study using a different adjuvant, RIBI (Rabbit 33088) or same adjuvant (Rabbits E6345 and E6370), and had been injected periodically for approximately 24 months prior to this study. For a separate project, ten rabbits (Mohican Valley Rabbitry, Loudenville, OH) not selected for atropinase activity (Group 5) had been immunized previously with atropine-PABA-BSA in Freund's adjuvant, and their antisera were included in selected portions of these characterization studies.

A total of 33 study rabbits and 10 other rabbits were immunized according to the schedule in Table 4 (Appendix D) . All rabbits were immunized over 144 days. The five best animals from Groups 1, 2, and 3 and one rabbit from Group 4 were selected for additional injections for a total of nine injections and five serum sample collections over a 284-day period. Group 5 animals were immunized for only 144 days.

Antisera Binding Activity. Each antiserum was evaluated for binding activity with atropine. For this assay, rabbit antiserum (100 μL) was diluted 1:100 (1:500 final dilution) in 1 percent heat-inactivated, normal rabbit serum and tested in the atropine RIA, substituting the diluent for the atropine standards. The percent total bound counts (%B/T) were determined and used to rank each antiserum.

There was considerable variation in antibody response to the different atropine-protein conjugates (Figure 10, Appendix C). Only six of the study rabbits demonstrated binding activity ≥50 percent (85058, 94248, 84835, 85340, 85487, and 33088). A significant response (≥20 percent binding) was obtained for five rabbits in both Group 1 and Group 3, all three rabbits in Group 4, but none of the Group 2 rabbits. For the rabbits receiving the immunogen in Freund's adjuvant, small lesions were obtained at the injection sites. This is typical with this adjuvant. However, the Hunter's TITERMAX™ produced large, necrotic areas varying in size from 3 to 10 centimeters in length. After discussions with the manufacturer, the adjuvant was discontinued and only the antigen in saline was continued. After the lesions resolved (approximately Day 144), use of the Hunter's TITERMAX™ was resumed at one-tenth the original amount in five selected rabbits, and no additional lesions were observed.

Antisera binding activities from the five best rabbits in both Group 1 and Group 3 were plotted against time to assess the kinetics of the anti-atropine response (Figure 11, Appendix C). The antibody levels increased

significantly for most rabbits after five injections, and appeared to plateau at approximately Day 144. Additional injections did not produce significant increases in binding activity. There were no apparent differences in the kinetics of antibody formation between Groups 1 and 3.

Antisera Titer. After determining the binding activity, the antisera titers of the individual rabbits in each group were determined for antisera collected on Day 144. These sera were selected for analysis because they represented the last samples for all rabbits. Twofold serial dilutions of the rabbit antisera, starting at 1:100, were prepared in 1 percent heat-inactivated, normal rabbit serum in PBS and then used in the binding assay. The titer was defined as the reciprocal of dilution nearest to 50 percent maximal binding. The mean titer and standard deviation were calculated for each group.

Group 3 had the highest mean titer of 620 ± 546 (Table 5, Appendix D), while essentially no response was obtained for Group 2. These results are consistent with the binding data obtained. For individual animals, high titers ($\geq$1:800) were obtained for four animals in Group 3, two animals in Group 1, two animals in Group 5, and one animal in Group 4. Statistical analyses were performed to determine if there were significant differences among the immunization groups. The antibody bindings of the various groups were compared using ANOVA nonparametric analyses. Group 2 was significantly different from the other groups using the Kruskal-Wallis one-way ANOVA, but the other group comparisons were equivalent ($p = 0.141$). These results suggest that the use of neostigmine with the atropine-PABGA-TG and Freund's adjuvant did not improve antibody formation. Furthermore, unselected rabbits (Group 5) gave the second highest mean titer (350 ± 254), indicating that selection of rabbits free of serum atropinase was not necessary. Subsequently, frozen sera from all Group 5 rabbits (N = 10) were tested for atropinase activity and all rabbits were positive except for Rabbit 3. Overall, the rabbits having the best anti-atropine titers were 84835, 33088, 85487, 85058, 85301, 9, and 1. Further analyses of these rabbit antisera in the atropine RIA were limited to five antisera (84835, 85487, 85058, 33088, and 9) because of the complexity of the RIA and specificity analysis. Rabbits from different immunization groups were selected to allow for further comparisons of their characteristics in the atropine RIA.

Optimization of Antisera Dilution in Atropine RIA. Following the antisera titration, the optimal dilution was determined for the standard curves using the improved conditions described for the automated atropine RIA. Anti-atropine sera at three dilutions were tested, and optimal assay characteristics for sensitivity and response range were determined. The following performance characteristic goals were used to select the optimal antisera dilution: sensitivity ≤1 ng/mL (≤50 pg/50 μL), response range of 1-100 ng/mL (50-5000 pg/50 μL), slope ≥0.980, y-intercept of 5.975, ED_{50} of 8.6 ng/mL (432.7 pg/50 μL), B/T value ≥0.30, and correlation coefficient ≥0.990. Using these criteria, the optimal antisera dilutions were 1:4,000 for 84835, 1:1,000 for 85487, 1:1,000 for 85058, 1:4,000 for 33088, and 1:2,000 for 9. A representative RIA standard curve for optimized antisera dilutions is shown for Rabbit 84835 in Figure 12 (Appendix C). In general, the inhibition curves exhibited loss of response at the lower atropine concentrations due to low affinity antibodies, which results in decreased precision, while at higher dilutions, the slope of the inhibition curve was decreased, resulting in a loss of response at the extremes of the curve. For comparison, the WRAIR antisera were used at a dilution of 1:1600 in previous studies. The antisera from the present study were diluted an additional fivefold, resulting in final dilutions ranging from 1:5000 to 1:20,000, and results were comparable to a 1:8000 final dilution for the WRAIR antisera.

Assay Characteristics. In addition to titer, other atropine RIA parameters were compared using standard curves for each antiserum. These assay characteristics were equivalent for R/T, B/T, slope of the linear regression line, correlation coefficient, and ED values (Table 6, Appendix D). However, these antisera produced a detection limit of 12.5 pg/50 μL (0.25 ng/mL), which is sixfold lower than that for the WRAIR antisera (75 pg/50 μL or 1.5 ng/mL). The inhibition curves also had a greater response range than that for the WRAIR antisera, extending linearly from 12 to 3200 pg/50 μL or 0.25 to 64 ng/mL. Part of this difference, however, may be attributed to the changes made in the atropine RIA procedures at the beginning of this project.

The average binding constant for each antiserum was determined. Scatchard plots of the B/F and nM bound atropine were plotted, and the average binding constant was calculated from the slope of the linear regression line. The values for the binding constant for the five antisera ranged from

0.05-0.14 nM^{-1} (Table 6, Appendix D). This binding constant is twofold to fivefold lower than that for the WRAIR antisera (0.28 nM^{-1}).

The percent coefficient of variation (%CV) was also evaluated over the response range of the standard curve. The %CV values were typically <4 percent (Table 7, Appendix D) and ranged from 0.2 to 3.1 percent for Rabbit 84835, 0.4-3.9 percent for Rabbit 85487, 0.3-3.4 percent for Rabbit 85058, 0.2-3.7 percent for Rabbit 33088, and 0.4-10.8 percent for Rabbit 9. These data are comparable to those for the WRAIR antisera (1.0-5.1 %CV).

For the four study rabbits, a total of 120.2 mL of high-titered antiserum was collected (Table 8, Appendix D). Using the appropriate antiserum dilution, this volume corresponds to over 1,500,000 tests.

Specificity Studies. The binding specificity of the antibodies was determined using the optimized antibody dilution in the automated atropine RIA. Inhibition curves for various compounds were determined using concentrations 100-fold greater than the 50 percent inhibition concentration for atropine. The following compounds were evaluated for crossreactivity in the atropine RIA: l-hyoscyamine hydrochloride, d,l-homatropine hydrobromide, (-) scopolamine hydrochloride, acetylcholine iodide, atropine methylnitrate, tropine, and d,l-tropic acid. Atropine methylnitrate and scopolamine (hyoscine) have modified tropine moieties, while homatropine has mandelic acid substituted for tropic acid. Crossreactivity of tropine and tropic acid must be ≤5 percent since they are the principal metabolites of atropine. However, crossreactivity ≤20 percent is acceptable for the other compounds, since they are not normally present in clinical samples or atropine preparations.

Compounds were evaluated at 0.2, 2, 20, and 200 nM. These concentrations were selected to provide concentrations on the atropine inhibition curve at 10- and 100-fold higher levels than the approximate midpoint of this curve. Inhibition curves for Rabbit 85487 antisera are representative of the reactivity with these compounds (Figure 13, Appendix C). The ED_{50} and percent crossreactivity are summarized in Table 9 (Appendix D). Scopolamine, acetylcholine iodide, atropine methylnitrate, tropine, and tropic acid showed no significant crossreactivity (≤3 percent) with the atropine antisera at the concentrations tested. However, l-hyoscyamine and d,l-homatropine showed significant inhibition in the atropine RIA. The ID_{50} of l-hyoscyamine and d,l-homatropine ranged from 0.2-2 nM and 0.6-3.6 nM,

respectively, resulting in crossreactivity of 13 percent to 50 percent and 5 percent to 50 percent, respectively. Specificity results for these atropine antisera were similar to those for the WRAIR antisera except for a higher crossreactivity with l-hyoscyamine for antisera prepared using both conjugates, and also a higher crossreactivity with d,l-homatropine for the antisera against the atropine-PABGA-TG conjugate.

3.2.3 Conjugate Degradation Studies

Background. Atropine-protein conjugates in rabbits are exposed to various esterases that could degrade the atropine, resulting in tropic acid. The enzymatic degradation of atropine in rabbits is well established.[1] Atropine has an *in vivo* half-life of 4 hours.[18] A study was conducted to evaluate the stability of atropine-protein conjugates to rabbit serum and liver esterases. Since the actual levels of esterase enzymes in serum and various cellular compartments can only be approximated, atropine degradation was used to establish the reaction conditions for treating the conjugates with these enzymes.

Approach. Atropine-PABA-TG and atropine-PABGA-TG at 900 nM atropine equivalents were treated with rabbit serum esterase and rabbit liver esterase. Atropine equivalents of the conjugates were estimated from the atropine-protein conjugate characterization. The esterase degradation conditions were established by treating atropine with various amounts of these enzymes and monitoring the production of tropic acid by HPLC with a C_{18} column using UV absorption (Figure 14, Appendix C)). The retention time of atropine is ~20.56 min, while that of tropic acid is ~8.18 min. The optimal conditions for >90 percent degradation of 90 μM atropine were 50 percent rabbit serum and 1 mg/mL rabbit liver esterase at 25°C for 18 hr.

Degradation studies were performed for the atropine conjugates. Atropine was used at 90 μM levels because of the detection limit of the HPLC detectors for atropine and tropic acid. In the RIA, conjugates as well as atropine (positive control) were treated with enzyme at 9 μM and tested at 0.2, 2, 20, and 200 nM. These studies revealed that the conjugates as well as atropine were degraded by these enzymes (Table 10, Appendix D). The atropine-PABGA-TG was approximately fourfold more susceptible to rabbit serum esterase

degradation than atropine-PABA-TG. This difference may be attributed to the longer crosslink provided by the PABGA. Although the rabbit liver esterase degraded the atropine, it did not produce significant degradation of either conjugate. The inhibition ELISA indicated that only a fraction of the atropine molecules attached to the carrier protein are hydrolyzed to tropic acid. These studies only approximate the conditions within an animal, and further studies would be needed to evaluate the actual degradation of the atropine conjugates by esterases in rabbits.

4.0 CONCLUSIONS

Three approaches were evaluated for increasing anti-atropine antibody titer in rabbits. These were a high-density conjugate, an alternative adjuvant, and an esterase inhibitor. High-titer antisera (≥1:800) were obtained in several groups. The highest mean titer was obtained in animals receiving the high-density atropine conjugate (atropine-PABGA-TG) in Freund's adjuvant with neostigmine. However, the group mean titers were not statistically different from those in animals immunized with a lower density conjugate (atropine-PABA-BSA). In addition, rabbits not screened for plasma atropinase produced a response equivalent to rabbits that were atropinase free. The unscreened rabbits were subsequently tested for atropinase activity and of these 10 animals, all were atropinase positive except Rabbit 3.

Although the atropine density and atropinase status did not appear to be significant factors in antibody formation, the number of injections and the adjuvant were important. The antibody response to atropine was optimal after five injections with either of the atropine conjugates. Furthermore, Freund's adjuvant produced a better response than the TITERMAX™. Although the TITERMAX™ adjuvant was advertised to reduce the necrotic lesions created by Freund's adjuvant, severe cutaneous lesions were observed in most of the animals receiving this adjuvant. Only small cutaneous lesions (0.5-2 cm) appeared on animals injected with Freund's adjuvant, and the highest antibody response was obtained with these groups. Adjuvants are typically used to enhance antibody production by providing a nonspecific stimulus to the animal's immune system and to prolong release of the antigen from subcutaneous and intramuscular injection sites to local lymph nodes containing the immune cells (macrophages,

T-lymphocytes, and B-lymphocytes) for antibody formation. Freund's adjuvant contains light mineral oil and Arlacel A for incomplete adjuvant and heat-killed Mycobacterium butyricum for complete adjuvant.[20] Hunter's TITERMAX™ consists of squalene with a block copolymer CRL89-41 attached to microparticulate silica.[21] In a previous task, RIBI adjuvant consisting of monophosphoryl lipid A with trehalose dicorynomycolate was used and the antibody titers were equivalent to those obtained using Freund's adjuvant, but no lesions were produced.

The magnitude of individual animal antibody response to the atropine conjugates varied significantly. This variation may be attributed to genetic differences. Of the five best responders, two were atropinase-negative rabbits with the high-density conjugate in Freund's adjuvant with neostigmine (Rabbits 84835 and 85487), one was an atropinase-negative rabbit with high-density conjugate in Freund's adjuvant with no neostigmine (Rabbit 85058), one was an atropinase-negative rabbit with low-density conjugate in Freund's adjuvant with no neostigmine (Rabbit 33088), and one was a rabbit of unknown atropinase status given low-density conjugate in Freund's adjuvant with no neostigmine (Rabbit 9). Frozen serum from Rabbit 9 was subsequently tested for atropinase activity and was positive. The rabbits receiving neostigmine did appear to respond more rapidly than the others, but the magnitude and duration of the response were similar to those of other responding groups. Once the antibody response plateau was achieved, the antibody titer did not increase with continued booster immunizations. The extended immunization schedule allowed for continued collection of rabbit antisera from the four responding rabbits, resulting in a total volume of 120 mL, corresponding to 1,500,000 tests in the atropine RIA.

Another possible explanation for low antibody titer production is chemical instability of the atropine molecule. Atropine is an ester of tropine and tropic acid. During antibody formation, the atropine conjugate is phagocytized by macrophages to form intracellular endosomes. These subcellular organelles have an internal pH of 4-5. During antigenic processing, endosomes fuse with lysosomes containing various degradative enzymes, including esterases.[16,17] Thus, the low pH and esterase content of the endosomes can result in cleavage of the atropine ester linkage, leaving tropic acid bound to the carrier protein and releasing free tropine. Therefore, low antibody titer

of animals may be attributed to the chemical instability of atropine conjugated to carrier protein. Studies using rabbit serum and liver esterases were performed to degrade atropine. The atropine-PABA-TG retained 70-80 percent of the antibody-reactive atropine, while the atropine-PABGA-TG was decreased fivefold by the rabbit serum but not by the rabbit liver esterase. Inhibition ELISA results indicated that only a small fraction of the atropine molecules were hydrolyzed to tropic acid when attached to a conjugate. Approximately 20-30 percent of rabbits are naturally atropinase-free, and such rabbits may have been used in the atropinase-unselected group. However, serum atropinase levels did not appear to be a significant factor in antibody response. This may be attributed to the adjuvant emulsion preventing access to the atropine attached to the protein.

The Battelle antisera were compared to the WRAIR antisera for use in the atropine RIA. Historical data for the WRAIR antisera were used in this evaluation. The optimal dilution of the antibody ranged from 1:1000 to 1:4000 for the Battelle antisera, which was equivalent to that of the WRAIR antisera (1:1600). The percent binding at these dilutions were 41 to 58 percent for the Battelle antisera and, again, were similar at 57 percent for the WRAIR antisera. Other characteristics of the RIA, such as slope, y-intercept, and correlation coefficient of the linear regression line, were also comparable between these antisera. However, the Battelle antisera consistently achieved a detection limit of 12.5 pg/50 μL (0.25 ng/mL), that is, approximately sixfold lower than that for the WRAIR antisera (75 pg/50 μL or 1.5 ng/mL). The 20, 50, and 80 percent inhibition doses for the Battelle antisera were also proportionally lower. The response range was extended from 75-1000 pg/50 μL (1.5-20 ng/mL) for the WRAIR antisera to 12-3200 pg/50 μL (0.25-64 ng/mL) for the Battelle antisera. These differences may be due to improvements made in the atropine RIA, rather than differences in antisera. Changes to the RIA procedures included increasing the amount of rabbit serum to 150 μL, making the standards from one stock and at a constant volume of 50 μL in 1 percent heat-inactivated NRS in PBS, changing the ammonium sulfate precipitation conditions to overnight at 4°C, reducing the atropine-antibody incubation to 1 hr at room temperature, and increasing the liquid scintillation counting time to 10 min or until a preset value of 2 percent CV was obtained. These changes led to a highly reproducible assay with <4 %CV over the response range.

The antisera were evaluated for crossreactivity with a group of structurally related compounds. The atropine metabolites, tropine and tropic acid, produced no crossreactivity at concentrations ranging from 0.2 to 200 nM. These results are consistent with findings of Wurzburger, et al.,[1] and are significant in that the atropine RIA will measure the atropine parent molecule but not the major metabolic products. Crossreactivity was obtained with l-hyoscyamine (13-50 percent) and d,l-homatropine (5-50 percent) at the ID_{50} level. l-Hyoscyamine is an l-isomer of atropine, and d,l-homatropine has a hydroxy group in place of the methylhydroxy group on the tropic acid moiety. Although these structural differences are minor, twofold to tenfold more compound was needed to obtain a binding equal to that of atropine. Similar results were obtained by Wurzburger, et al. Antisera to atropine-PABA-BSA did not significantly crossreact with d,l-homatropine, as did the atropine-PABGA-TG antisera (5 to 6 percent vs. 21 to 50 percent, respectively). This result implies that the shorter-crosslink compound, PABA, may result in more specific antibodies. Although these data are of interest, they are not significant in the clinical application of the method since neither compound is anticipated to be present in plasma samples. Scopolamine and atropine methylnitrate are also structurally similar to atropine, with modifications to the tropine moiety with an epoxide added to the ring structure or a methylnitrate at the amine group of the tropine moiety, but with an identical tropic acid moiety. Neither compound crossreacted significantly with the Battelle atropine antisera. Likewise, acetylcholine iodide did not crossreact. These results suggest that the structural differences in the tropine moiety change the immunoreactivity dramatically. In addition, the crosslink compound may crossreact with the tropic acid moiety.

In conclusion, additional anti-atropine sera of high quality and sufficient quantity was produced for future pharmacokinetic studies. The automated RIA method also was improved, resulting in greater reliability and improved assay precision. Performance criteria for detection limit, response range, precision, and specificity were met or exceeded, thus providing a method with experimentally useful assay characteristics for pharmacokinetic studies. The atropine RIA characteristics of the Battelle antisera were comparable to those of the WRAIR antisera, and were consistent with the plasma/serum atropine concentrations anticipated in future pharmcokinetic studies.

5.0 RECOMMENDATIONS

The quantity and quality of the anti-atropine antibody produced is sufficient for sample analyses by RIA and is equivalent to that obtained by Dr. Smallridge of WRAIR. The atropine conjugate analysis using ELISA indicates that this nonisotopic immunoassay has potential advantages over the atropine RIA. Potential advantages would be a lower detection limit (10 pg/mL), broader response range (15,000-fold), more efficient use of atropine antisera (≥1:10,000-fold dilution), higher throughput assay format, and elimination of the use of radioisotopes and its associated waste. Studies with current antisera could be performed to demonstrate these improvements and to validate this nonisotopic method.

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The names, role in the study, and highest academic degree of principal contributors in this study are presented in the following list.

Name	Title	Degree
Larry S. Miller	Study Director	Ph.D.
Balwant S. Bhullar	Task Leader	Ph.D.
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7.0 REFERENCES

(1) Wurzburger R.J., R.L. Miller, H.G. Boxenbaum, and S. Spector. 1977. Radioimmunoassay of atropine in plasma. J. Pharm. Exp. Ther., 203:435-441.

(2) Kradjan, W.A., R.C. Smallridge, R. Davis, and P. Verma. 1985. Atropine serum concentrations after multiple inhaled doses of atropine sulfate. Clin. Pharmacol. Therap., 38:12-19.

(3) Mooz, E.D. Amino acids. Practical Handbook of Biochemistry and Molecular Biology (edited by G.D. Fasman). CRC Press, FL. 1989. pp. 323.

(4) Peters, T. Serum Albumin. In Advances in Protein Chemistry (edited by C.B. Anfinsen, J.T. Edsall, and F.M. Richards). Academic Press, NY. 1985. 37:176.

(5) Mooz, E.D. Amino acids. Practical Handbook of Biochemistry and Molecular Biology (edited by G.D. Fasman). CRC Press, FL. 1989. pp. 182.

(6) Little, J.R. and H. Donahue. Spectral properties of proteins and small molecules. Methods in Immunology and Immunochemistry (edited by C.A. Williams and M.W. Chase). Academic Press, NY. 1968. Vol 2, p. 352.

(7) Voller, A., A. Bartlett, and E. E. Bidwell. 1978. Enzyme immunoassays with special reference to ELISA techniques. J. Clin. Path., 31:507-520.

(8) Fliniaux, M.A., F. Manceau, and A. Jacquin-Dubreuil. 1993. Simultaneous analysis of l-hyoscyamine, l-scopolamine, and dl-tropic acid in plant material by reverse-phase high-performance liquid chromatography. J. Chromatog., 644:193-197.

(9) Makela, O. and I.J.T. Seppala. Haptens and carriers. In Handbook of Experimental Immunology (edited by D.M. Weir, L.A. Herzenberg, C. Blackwell, and L.A. Herzenberg). Blackwell Scientific Publications, CA. 1986. Vol. 1:3.1-3.13.

(10) Chase, M.W. Immunization procedures. In Methods in Immunology and Immunochemistry (edited by C.A. Williams and M.W. Chase). Academic Press, NY. 1967. Vol 1, p. 213.

(11) Brown, N.D. and H.K. Sleeman. 1978. Ion-pair high-performance liquid chromatography method for the determination of atropine sulfate and tropic acid. J. Chromatog., 150:225-228.

(12) Merken, L., M.J. Simons, S. Swillens, M. Massaer, and G. Vassart. 1985. Primary structure of bovine thyroglobulin deduced from the sequence of its 8,431-base complementary DNA. Nature, 316:647-651.

(13) Deutsch, H.F. Antigens. In Methods in Immunology and Immunochemistry (edited by C.A. Williams and M.W. Chase). Academic Press, NY. 1967. Vol 1, p. 155.

(14) Mooz, E.D. Amino acids. In Practical Handbook of Biochemistry and Molecular Biology (edited by G.D. Fasman). CRC Press, FL. 1989. pp. 3-68.

(15) J.R. Dias and G.R. Pettit. 1971. Reduction of δ-lactones and hindered esters with diborane. J. Org. Chem., 36:3485-3493.

(16) McCluskey, J., R. Blok, A. Brooks, W. Chen, D. Kanost, and L. Kjer-Nielsen. The biology of antigen processing and presentation. In Antigen Processing and Recognition (edited by J. McCluskey). CRC Press, FL. 1991. pp. 1-54.

(17) Nelson, D. S. Immunobiology of Macrophages. Academic Press, NY. 1976. pp. 418-421.

(18) Brown, B. Atropine, scopolamine, and related antimuscarinic drugs. In Goodman and Gilman's The Pharmacological Basis of Therapeutics. Eighth Edition. Pergamon Press, New York. 1990. pp. 151-165.

(19) Wasserman, N.H., A.S. Penn, P.I. Freimuth, N. Treptow, S. Wentzel, W.L. Cleveland, and B.F. Erlanger. 1982. Anti-idiotypic route to anti-acetylcholine receptor antibodies and experimental myasthenia gravis. Proc. Natl. Acad. Sci. USA, 79:4810-4814.

(20) Deutsch, H.F. Conjugated and synthetic antigens. In Methods in Immunology and Immunochemistry (edited by C.A. Williams and M.W. Chase). Academic Press, NY. 1967. Vol 1, p. 124.

(21) Bennett, B., I.J. Check, M.R. Olsen, and R.L. Hunter. 1992. A comparison of commercially available adjuvants for use in research. J. Immunological Methods, 153:31-40.

APPENDIX A

PROTOCOL

Development of Procedures to Enhance Atropine Antisera Production in Rabbits

Study Performed by Battelle Memorial Institute,
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6. Introduction: A primary mission of the U.S. Army Medical Chemical Defense Program is to develop improved treatment regimens for nerve agent poisoning. Determination of the pharmacokinetics of an improved treatment regimen's components (atropine and oxime) is a critical portion of the submission to the U.S. Food and Drug Administration for approval to field the regimen. The current acceptable method for measuring atropine levels in blood (database from which atropine pharmacokinetic parameters are generated) is a radioimmunoassay (RIA) method which requires atropine antisera of acceptable titer. To support anticipated projects, additional atropine antisera must be generated. However, current attempts to generate atropine antisera using information from published reports and interviews on this topic are significantly below the target antisera titers (current 1:100 and target 1:1600). The purpose of this task is to investigate several approaches which may enhance atropine antisera formation in rabbits.

7. Objective: The objective of this investigation is to evaluate alternative approaches to production of adequate titers of atropine antisera in rabbits. This project will be conducted in two sequential phases. Phase I will be the preparation of three different atropine conjugates. In Phase II, antisera will be prepared in rabbits by repeated injections of the different atropine conjugates and their characteristics will be compared to antisera from Dr. Smallridge at the Walter Reed Army Institute of Research (WRAIR) using the atropine radioimmunoassay.

   An organic compound such as atropine does not normally elicit antibody formation, but can be rendered immunogenic in rabbits by coupling the atropine molecule to a relatively high molecular weight (MW) carrier

protein (hapten). Atropine has been conjugated with bovine serum albumin (BSA; M.W. 67,000) at a level of approximately 30 residues of atropine per molecule of BSA. This conjugate (BSA-atropine) has been used as an immunogen to create antibodies in rabbits. Although antisera were usable in a RIA for atropine, the titers were low (1:20 to 1:100) relative to WRAIR antisera (1:1600 titer). Conversations with WRAIR scientists who produced the high titer antisera (Drs. Smallridge and Verma) revealed that only approximately 1 in 8 rabbits produce a high titer antiserum ($\geq$ 1:1000). Rabbits are often chosen for antisera production because of their normally strong response to antigen conjugates. The most likely reason for the low titer antisera previously obtained is the instability of the ester bond in the atropine moiety - the ester linkage between tropine and tropic acid to form atropine is susceptible to hydrolysis at high pH and by various esterases. A significant improvement in immunogenicity may be achieved by increasing the number of atropine residues that are covalently attached to the carrier protein and altering the conjugation chemistry to avoid pH extremes.

8. Experimental Design:

A. Test System

(1) Animals - Approximately 40 female, atropinase-free New Zealand White rabbits will be procured from Hazleton Research Animals, Denver, PA, or equivalent source, for use in this study. Rabbits are routinely used for the production of serum antibodies against many antigens, and rabbits have been used to produce antibodies for atropine in previous studies at this laboratory. The rabbit has been shown to produce atropine antibodies, can provide sufficient blood volumes for determining antibody titers and for harvesting antibodies, and can be easily maintained in a controlled environment in the numbers necessary for determining efficacy of various antigen conjugates in provoking an antibody response.

(2) Initial Weight - When obtained, rabbits will weigh approximately 1.5-3.0 kg.

(3) Quarantine - Rabbits are held in isolation and observed for signs of clinical illness for at least 7 days prior to study initiation. (Battelle SOP ARF. II-019)

(4) Animal Selection - Each animal will be examined and its health status assessed by a veterinarian within 48 hours of receipt. Each animal will be tested for serum atropinase in accordance with procedures of MREF Method No. 1/General, and only atropinase negative animals will be used in this study.

(5) Animal Identification - All animals are tagged in the ear to retain positive identification. Cage cards will be color-coded by treatment group.

(6) Housing - Animals are housed individually in stainless-steel, slotted cages equipped with automatic watering systems.

(7) Lighting - The light/dark cycle will be approximately 12 hours each per day, using fluorescent lighting. (Battelle SOP ARF. III-010)

(8) Temperature - The room temperature will be maintained at 61 to 72 F and will be recorded twice daily. At least 90 percent of the total recordings will fall within the specified range. (Battelle SOP ARF. III-007)

(9) Humidity - The relative humidity of rooms housing rabbits will be maintained at 40 to 60 percent and readings will be recorded twice daily. At least 90 percent of the total readings will be within the specified range. (Battelle SOP ARF. III-007)

(10) Diet - Upon receipt all animals will be fasted for 24 hours. The amount of feed (certified Purina Rabbit Chow) will then be increased over a 5-7 day period to a maximum of 100-125 grams of food per day. Analysis of each feed lot can be obtained from the vendor. There are no known contaminants in the feed that would interfere with the purpose or conduct of the study. (Battelle SOPs ARF. II-014, ARF. II-017, and ARF. III-004)

(11) Water - Water is supplied from the Battelle water system and given ad libitum during quarantine and holding. No contaminants that would affect the results of the study are known to be present in the water. (Battelle SOPs ARF. II-025 and ARF. III-003)

(12) Laboratory Animal Welfare Practices - Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-R-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurance regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health on August 27, 1973. Animals for MREF studies are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication

No. (NIH) 85-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966 as amended (P.L. 89-544 and P.L. 91-579).

(13) Accreditation - On January 31, 1978, Battelle's Columbus Division received full accreditation of its animal care and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

(14) Disposition of Experimental Animals - Rabbits producing a high titer of atropine antibodies may be maintained indefinitely. Rabbits that do not produce high serum atropine antibody titers will be euthanized with an approved intravenous euthanasia solution and cremated.

B. Test Material - Supplies, as needed, will be purchased from chemical supply houses or provided by the U.S. Army.

C. Experimental Approach - Three separate approaches will be made in an effort to create higher serum antibody titers for atropine in rabbits:

(1) Approach 1 (High Density Conjugate) - An atropine-protein conjugate will be synthesized using a high density protein as the carrier protein and conditions that avoid pH extremes. For example, thyroglobulin has approximately 2.5 fold more available sites for coupling than BSA. Therefore, it should be possible to couple 100-150 residues of atropine to each molecule of thyroglobulin (M.W. 669,000). This will ensure that sufficient atropine residues remain attached to the carrier protein following its injection into an animal. Porcine thyroglobulin has been successfully used as the carrier protein for small organophosphorus compounds (M.W. 290-310) and produced an enzyme-linked immunosorbent assay (ELISA) titer of over 1:100,000. Conjugation with other high density proteins is possible, therefore, selection of the protein to be used will be made in collaboration with Army immunologists.

The conjugation chemistry described, and originally used by Wurzberger et al.[1], will be modified to prevent generation of a low or high pH that might result in cleavage of the atropine molecule at the ester site. Para-aminobenzoic acid (PABA), a heterobifunctional molecule, will be used to crosslink atropine to the thyroglobulin carrier protein.

The conjugates will be characterized for atropine incorporation. The trinitrobenzene sulfate (TNBS) reaction[2] will be used to determine the number of remaining free amino groups after the reaction. Infrared (IR) spectroscopic analysis will detect esters and ethers present in the atropine molecule or its analogue. The tertiary amine of the tropine moiety will be quantitated by a platinum chloride and potassium iodide reaction[3]. ELISA testing for antigenicity will be accomplished with atropine-BSA antisera. There should be 50-150 residues of atropine per thyroglobulin molecule for the conjugate to be acceptable for antisera production in Phase II. The conjugate having the highest atropine incorporation will be used.

(2) Approach 2 (Atropine Analogue) - One approach to eliminate atropine hydrolysis is to convert the atropine ester linkage to an ether linkage. Atropine will be converted to an ether derivative using diborane in tetrahydrofuran (THF) as the reagent for the reduction of the ester function. The selectivity of this reagent is very dependent on the structure of the ester; it has been used to convert sterically hindered esters such as the steroidal (lanostanyl) ester of pivalic acid to the corresponding neopentyl ester[4]. Modifications of this approach, if required, may lead to increased quantities of ether compared to alcohol products. Use of sodium borohydride/boron trifluoride etherate (which produces diborane) gives higher ether:alcohol products in comparison to using diborane/boron trifluoride etherate on the same ester substrate[5]. This suggests that the borohydride anion can selectively participate in ether formation. The ether:alcohol ratio can also be influenced by the proportion of diborane to ester substrate.

The reduction of atropine will be initially investigated using diborane in THF under a range of conditions. The reaction times, reaction temperatures and number of equivalents of diborane will be varied to maximize conversion to the atropine ether. If low conversion rates are obtained, use of alternative reagents such as sodium borohydride/boron trifluoride etherate will be investigated. These reactions will be monitored using analytical gas chromatography (GC), Thin Layer Chromatography (TLC), or High Performance Liquid Chromatography (HPLC) techniques. The alcohol cleavage products, tropine and 2-phenylpropane-1,3-diol, will be

purchased and used to help determine the most appropriate analytical method for following these reactions. Proton and ^{13}C nuclear magnetic resonance (NMR) spectroscopy can also be used to distinguish between the two potentially competing pathways during method development.

Preliminary purification of the desired product will be performed with preparative HPLC or preparative TLC. The ether derivative is probably a solid and may be further purified by recrystallization. Alternatively, converting the ether derivative to its oxalate or hydrochloride may be used for purification purposes; this would also stabilize the material for storage purposes. Although the yield of ether derivatives may be low (< 10 percent), adequate quantities (200-300 mg) may be obtained for conjugation.

The final structure identification will be performed by proton and ^{13}C NMR spectroscopy, IR spectroscopy and elemental analysis. Melting points will also be obtained as part of the characterization of this compound. The atropine analogue will be conjugated to thyroglobulin using the coupling chemistry described in Approach 1 and the conjugate used for antisera production in Phase II.

(3) Approach 3 (Esterase Inhibitor) - Atropine is an ester of tropic acid and tropine. This ester is susceptible not only to chemical cleavage but also enzymatic hydrolysis. Rabbits and other species contain cholinesterases and other esterases in blood and cells which can degrade atropine. Therefore, the addition of an esterase inhibitor may decrease atropine degradation.

An esterase inhibitor such as neostigmine is active in biological systems and has a chemical structure similar to atropine. The esterase inhibitor at a 0.2 mM final concentration[6,7] will be mixed with 1 mg of atropine-protein conjugate in an adjuvant and administered to rabbits. Assuming 100 molecules of atropine are incorporated into each thyroglobulin molecule, this is over a 1,400 fold molar excess of neostigmine to atropine conjugate. As the adjuvant-antigen mixture is taken up by macrophages and other antigen-presenting cells, it is anticipated that the inhibitor will react with esterases and slow enzymatic degradation of atropine coupled to the protein but still allow the atropine-protein conjugate to be processed and presented by the accessory cell to T-helper lymphocytes and B-lymphocytes, resulting in antibody formation.

D. Antisera Production and Characterization

(1) Antisera Production

Rabbits will be used for the production of anti-atropine antibodies. Previously, Freund's adjuvant and RIBI (Hamilton, MT) adjuvant were used and produced equivalent results. We will continue to use Freund's adjuvant and an additional group of animals will receive Hunter's TITERMAX (CytRx Corporation, Norcross, GA) which has been touted as superior to Freund's adjuvant. Antigens to be used for these immunizations are high density atropine-thyroglobulin conjugate, atropine analogue-thyroglobulin conjugate, and atropine-thyroglobulin conjugate mixed with an esterase inhibitor.

Rabbits will be injected with 1-1.5 mL of antigen (approximately 1 mg of atropine-thyroglobulin conjugate), emulsified in adjuvant, at intramuscular and multiple subcutaneous sites (approximately 15-20 injection sites) every three weeks for 6 months. (Battelle SOP ARF. II-005) Animals will have hair clipped from injection sites prior to each immunization. (Battelle SOP ARF. II-007) Rabbits will be tested for atropinase activity prior to immunization according to the procedure outlined in MREF Method No. 1/General. Ten rabbits will be used for each antigen using Freund's adjuvant. In addition to these three groups, another group of ten rabbits will be injected using atropine-thyroglobulin conjugate with an esterase inhibitor and Hunter's TITERMAX adjuvant. Approximately 2-5 mL of blood will be collected from the medial artery of the ear (Battelle SOP ARF. II-022) on day 0 and at 6 weeks intervals for monitoring the anti-atropine response.

Antisera will be evaluated for titers by ammonium sulfate precipitation of antibody-^{3}H atropine complexes. For this assay, diluted rabbit antiserum (100 μL) will be mixed with 4,000-10,000 cpm ^{3}H-atropine in phosphate buffered saline (PBS) containing 10 percent normal rabbit serum (400 μL). After an incubation of 18 hrs at 2-8 C, 500 μL of saturated ammonium sulfate will be added to the reaction mixture and incubated for 15 minutes at room temperature to precipitate the ^{3}H-atropine-antibody complexes. The precipitate will be collected by centrifugation at 2,000 X g for 15 min at room temperature. The supernatant will be decanted and the pellet washed twice with 50 percent saturated ammonium sulfate. The pellet will be dissolved in 1 mL distilled

water and prepared for liquid scintillation counting using a water miscible counting solution (Hydrofluor®; National Diagnostics Inc., Manville, NJ). A Tecan RSP Model 5052 liquid handling system will be used to automate these titration studies in order to handle the large number of antiserum samples to be titrated. Because of variability in the rabbit response and low success rate with previous atropine-protein conjugates, the antiserum from each animal will be evaluated for titer.

The percent of bound radioactivity will be calculated for each dilution and antiserum titer reported as 50 percent maximum binding relative to WRAIR antiserum. By 6 months, the 4 rabbits having the highest titer will be selected for large volume bleedings to obtain adequate antisera for further characterization studies in the atropine RIA. The goal is to obtain atropine antisera with binding and specificity characteristics equivalent to, or better than, the WRAIR rabbit antiserum.

(2) Antisera Characterization - The antiserum from the selected rabbits will be evaluated in the atropine RIA. (Battelle SOP TOX VI-014) An optimum antiserum dilution will be defined and used to determine the detection limit, response range, $(B/T)_{max}$, $(B/T)_{o}$, and slope. Binding constants for each antiserum will also be determined using Scatchard or Sips plots of the competition curve data. The Tecan automated dispensing unit will also be used for these studies. The new antisera will be accepted only if the assay characteristics are equivalent or better than the WRAIR antiserum.

The binding specificity of the antibodies will be determined using the optimized antibody dilution in the atropine RIA procedure. Inhibition curves for various compounds will be performed using concentrations as high as 1000 fold greater than the 50 percent inhibition concentration for atropine. The following compounds will be evaluated for crossreactivity: l-hyoscyamine, atropine methylnitrate, scopolamine, homatropine, acetylcholine iodide, and metabolites of atropine (tropine and tropic acid). Atropine methylnitrate and scopolamine (hyoscine) have modified tropine moieties while homatropine has mandelic acid substituted for tropic acid. Crossreactivity of tropine and tropic acid must be $\leq$ 5 percent since they are the principal metabolites of atropine. However, $\leq$ 20 percent is acceptable for the

other compounds, since they are not normally present in the sample or atropine preparations. These inhibition studies will be performed using the previously developed, automated atropine RIA procedure. Again, the new antisera must have specificity equivalent to or better than the WRAIR antiserum to be acceptable for use in the atropine RIA.

9. Records to be Maintained: All records that would be required to reconstruct the study and to demonstrate adherence to the protocol will be maintained. This study will not be listed on Battelle's Good Laboratory Practices' (GLP) Master Study Schedule.

   Records and study material to be maintained will include:

   A. Quarantine Data
   B. Study Room Environmental Data
   C. Body Weights
   D. Immunization Records
   E. Bleeding Records
   F. Antigen Preparation Data
   G. Antisera Testing
   H. Atropine RIA Data

   All original study records will be retained in the Battelle archives or at the MREF. Upon acceptance of the final report, the remaining antigen preparations and antisera will be stored under conditions to maximize their long term activity ($\leq$ -70 C) for a period up to 5 years.

   If changes to the study protocol become necessary during its conduct, verbal agreement to make changes will be made among the Study Director, the MREF Manager, and the U.S. Army Contracting Officer's Representative (COR). Within 15 calendar days, any change and explanations will be documented in writing and formally approved by the Study Director, MREF Manager, and COR as an amendment to the study protocol.

10. Reports:

   A. Verbal monthly reports submitted on the 1st working day of each month during the project.

   B. Written quarterly reports submitted on the first working day of the month in which the quarterly report is due.

C. A Draft Final Report will be prepared within 30 calendar days after completion of all experimental and data analysis work. The Draft Final Report will include the following sections:

(1) Executive Summary

(2) Introduction

(3) Materials and Methods

(4) Results

(5) Conclusions

(6) Recommendations

(7) Acknowledgements

(8) References

(9) Signature page of key study personnel and their responsibilities.

D. Following receipt of Draft Final Report comments from USAMRDC, the Final Report will be prepared within 30 calendar days.

11. References:

1. Wurzberger et al, J. Pharmcol. Exp. Therap., 203:435, 1977.

2. Habeeb, A. F. S. A., Analyt. Biochem., 14:328, 1966.

3. Chakrin, L. W. and F. E. Shideman, Int. J. Neuropharmacol., 7:337, 1968.

4. Dias, J. R. and G. R. Pettit, J. Org. Chem., 36:3485, 1971.

5. Pettit, G. R. et al, Tetrahedron, 18:953, 1962.

6. Wasserman, N. H. et al., Proc. Natl. Acad. Sci., 79:4810, 1982.

7. Goodman and Gilman's Pharmacological Basis of Therapeutics, Permagon Press, NY. Eighth Edition, 1990. p. 139.

12. Approval Signatures:

David W. Hobson, Ph.D., D.A.B.T.
MREF Principal Investigator and Manager

9 SEP 92
Date

Larry S. Miller, Ph.D.
Study Director

15 Sept 1992
Date

Allan G. Manus, D.V.M.
Study Veterinarian

9/16/92
Date

LTC Don W. Korte, Jr., Ph.D.
COR, USAMRICD

24 SEP 92
Date

Garrett S. Dill, D.V.M.
Vice President
Product Registration and Evaluation

9/15/92
Date

Richard A. Shank
Quality Assurance Unit

9-15-92
Date

Development of Procedures to Enhance
Atropine Antisera Production in Rabbits

Protocol Amendment No. 1

Change: Page 7, Section 8, D.1. Antisera Production. Line 21 should be changed from:

"In addition to these three groups, another group of ten rabbits will be injected using atropine-thyroglobulin conjugate with an esterase inhibitor and Hunter's TITERMAX adjuvant."

To: "In addition to these three groups, another group of ten rabbits will be injected using atropine-thyroglobulin conjugate and Hunter's TITERMAX adjuvant. These rabbits will receive 0.4 mL of antigen (approximately 1.0 mg of atropine-thyroglobulin conjugate) emulsified in Hunter's TITERMAX, at four subcutaneous sites (approximately 0.1 mL per site) for the initial injection. Following that, each rabbit will receive 0.2 mL of antigen at four subcutaneous sites (approximately 0.05 mL per site) every three weeks for the remainder of the 6 months.

Reason: The reasons for this change are as follows: The esterase inhibitor is not used in the immunizations with TITERMAX, it is used in the conjugate mixed with Freund's adjuvant, as stated in Section 8, D.1. Antisera Production. Lines 7-9. The change in injection sites volume, and number of injections is due to the manufacturer's suggested protocol for using TITERMAX.

APPROVED BY:

Larry S. Miller, Ph.D.
Study Director — Date 12/2/92

David W. Hobson, Ph.D.
MREF Principal Investigator — Date 12/5/92

LTC Don W. Korte, Jr., Ph.D.
Sponsor's Representative — Date 12/10/92

QA Representative — Date 12/2/92

Development of Procedures to Enhance
Atropine Antisera Production in Rabbits

Protocol Amendment No. 2

Change 1: Page 7, Section 8.D.1. Antisera Production. The first sentence of the second paragraph is changed from:

"Rabbits will be injected with 1-1.5 mL of antigen (approximately 1 mg of atropine-thyroglobulin conjugate), emulsified in adjuvant, at intramuscular and multiple subcutaneous sites (approximately 15-20 injection sites) every three weeks for 6 months."

To: "Rabbits will be injected with 1-1.5 mL of antigen (approximately 1 mg of atropine-thyroglobulin conjugate), emulsified in adjuvant, at intramuscular and multiple subcutaneous sites (approximately 15-20 injection sites) for the initial immunization. Subsequent boosts will contain approximately 0.5-1.0 mg of antigen, and will be administered every three weeks for 6 months."

Reason: The reason for this change is to allow variation between the initial dose of antigen and subsequent exposure doses. The population of specific antibodies in the antisera will be enhanced if the amount of carrier protein (thyroglobulin) is reduced in booster injections.

Change 2: Page 7, Section 8.D.1. Antisera Production. The fifth sentence of the second paragraph is changed from:

"In addition to these three groups, another group of ten rabbits will be injected using atropine-thyroglobulin conjugate with an esterase inhibitor and Hunter's TITERMAX adjuvant."

To: "In addition to these three groups, another group of ten rabbits will be injected using atropine-thyroglobulin conjugate with an esterase inhibitor and Hunter's TITERMAX adjuvant. Also included in the immunization schedule will be three additional rabbits from Task 89-02 (G1555-0201) to be injected with atropine-bovine serum albumin conjugate. These rabbits will be boosted according to the schedule indicated in Change 1 above."

Reason: The reason for this change is to continue immunization of these rabbits (atropine-bovine serum albumin) as a source of high-titer anti-atropine antisera.

Change 3: Page 7, Section 8.D.1. Antisera Production. The last sentence of the second paragraph is changed from:

"Approximately 2-5 mL of blood will be collected from the medial artery of the ear (Battelle SOP ARF. II-022) on day 0 and at 6 week intervals for monitoring the anti-atropine response."

To: "Approximately 2-5 mL of blood will be collected from the medial artery of the ear (Battelle SOP ARF. II-022) of all study rabbits on day 0 (if applicable), and 10-13 days following alternate boosts, for monitoring the anti-atropine response."

Reason: The reason for this change is by collecting the blood 10-13 days after an injection the antibody levels will be higher due to restimulation of the immune system.

Change 4: Page 7, Section 8.D.1. Antisera Production. The first two sentences of the third paragraph are changed from:

"Antisera will be evaluated for titers by ammonium sulfate precipitation of antibody-^{3}H atropine complexes. For this assay, diluted rabbit antiserum (100 μL) will be mixed with 4,000-10,000 cpm ^{3}H-atropine in phosphate buffered saline (PBS) containing 10 percent normal rabbit serum (400 μL)."

To: "Antisera will initially be evaluated for titers by both the enzyme-linked immunosorbent assay (ELISA) and by the ammonium sulfate precipitation of antibody-^{3}H atropine complexes to establish equivalency between these methods. The ELISA will be performed using atropine-bovine serum albumin as the solid phase. Diluted rabbit antiserum will be allowed to bind, with the unbound portion washed off. Next, an enzyme-linked secondary antibody (anti-rabbit immunoglobulin) will be allowed to bind, with the unbound portion washed off. Finally, the appropriate substrate will be added, and the color change will be read on a microplate reader. For the ammonium sulfate precipitation, diluted rabbit antiserum (100 μL) will be mixed with 4,000-10,000 cpm ^{3}H-atropine in phosphate buffered saline (PBS) containing 10 percent normal rabbit serum (400 μL)."

Reason: The reason for this change is to establish the equivalency between the ELISA and RIA methods. In the future, ELISA may replace RIA since it is the faster of the two methods, taking less than one day, and it allows several (8-12) antisera to be titered on one assay plate, with the ability to run up to 10 assays per day. This is in

APPENDIX B

STANDARD OPERATING PROCEDURE FOR ATROPINE RIA

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Key Words: **ATROPINE, RADIO—IMMUNOASSAY, RIA**

Standard Operating Procedure (SOP)

THE DETERMINATION OF SERUM ATROPINE SULFATE CONCENTRATIONS BY RADIOIMMUNOASSAY (RIA)

Originator: Elizabeth J. Von Koskenbalz Date 2/18/94

Approved by: Joyce M. Dunphy for JSM Date 2/18/94
Technical/Product Line Manager

Approved by: R Alan Scantland Date 2/18/94
Business Unit Manager

Reviewed and Registered by QAU:
Cynthia Jean Tolson Effective Date 02-21-94

Distribution List:

Quality Assurance Unit
SOP Manual(s)

Battelle
505 King Avenue
Columbus, Ohio 43201

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I/II. SCOPE/PURPOSE:

The purpose of this Standard Operating Procedure (SOP) is to describe a Radioimmunoassay method employed in the determination of serum atropine sulfate concentrations.

III. REFERENCES:

1. Wurzburger, R. J., Miller, R. L., Boxenbaum, H. G., and S. Spector. 1977. Radioimmunoassay of Atropine In Plasma. J Pharmacol Exp Therap 203: 435.

2. Kradjan, W. A., Smallridge, R. C., Davis, R., and P. Verma. 1985. Atropine Serum Concentrations After Multiple Inhaled Doses of Atropine Sulfate. Clin Pharmacol Therap 38: 12.

IV. DEFINITIONS: None

V. PROCEDURES:

Preliminary Tasks

A. Preparation of Phosphate Buffered Saline (PBS), pH 7.5

1. Combine the following components to prepare 1 liter PBS (10 mM Na_2HPO_4, 150 mM NaCl), pH 7.5:

Na_2HPO_4	1.420 grams
NaCl	8.766 grams
distilled water	980.0 mL

2. Adjust the pH to 7.5 with 1 N HCl. Bring the volume to 1000 mL with distilled water.

3. Store PBS at 1-9°C. The PBS is stable for a period of one month from the date of preparation.

B. Preparation of Saturated Ammonium Sulfate

1. Combine the following reagents to prepare 500 mL saturated ammonium sulfate:

$(NH_4)_2SO_4$	257.6 grams
sterile distilled water (injection grade)	500.0 mL

2. Do not adjust pH. Store at 1-9°C. This reagent is stable for a period of one month from date of preparation. Prepare at least 24 hours prior to use.

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C. Preparation of 50 percent Saturated Ammonium Sulfate

1. Combined the following reagents to prepare 500 mL of 50 percent saturated ammonium sulfate:

$(NH_4)_2SO_4$	128.8 grams
distilled water	500.0 mL

2. Do not adjust pH. Store at 1-9°C. This reagent is stable for a period of one month from the date of preparation. Prepare at least 24 hours prior to use.

D. Preparation of ^{3}H-Atropine Stock Solution

1. ^{3}H-Atropine is prepared in PBS, pH 7.5 at a concentration of approximately 4000 CPM/20 μL. This material is aliquoted and stored at -70 ($\pm$5)°C. The labeled atropine is stable for a period of one year.

2. Thaw a fresh aliquot daily. Dispose of the leftover material at the conclusion of the experiment according to Battelle SOP for disposal of radioactive materials.

E. Preparation of Primary Atropine Stock Solution

1. Prepare a 1.0 mg/mL solution of atropine sulfate in PBS, pH 7.5. Weigh a minimum of 10.0 mg atropine sulfate. Mix thoroughly and aliquot. Store at -70 ($\pm$5)°C. The material is stable for a period of one year from the date of preparation.

F. Preparation of Rabbit Anti-Atropine Antisera Stock

1. The correct concentration of rabbit anti-atropine antisera will be determined in preliminary testing. The stock antisera is stored as 30 μL aliquots at -70 ($\pm$5)°C. Dilute the antisera to the proper concentration in 1% normal serum - PBS, pH 7.5. Prepare the diluted antibody fresh daily. Leftover material may be frozen and used for repeat analyses performed within a period of five days. Thereafter, dispose of the diluted material.

G. Normal Serum

1. A stock of normal serum from the same species as that of the serum samples being analyzed will be heat inactivated at 56°C for 30 min and then aliquoted and stored at -70 ($\pm$5)°C. The frozen stock is stable for a period of one year.

2. Aliquot(s) of normal serum are thawed freshly on the assay day. The serum is used undiluted in the assay. Unused material may be frozen and used on a subsequent test day.

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H. Diluent (1% Normal Serum - PBS)

1. Add 0.1 mL of heat-inactivated normal serum from same species as test samples to 9.9 mL PBS. Prepare on day of use and discard unused portion.

I. Test Samples

1. Test samples are stored at - 70 (±5)°C.

RIA Set Up (Day 1)

1. Prepare atropine sulfate Stocks 1 and 2 fresh daily from a freshly thawed aliquot of the Primary Atropine Stock solution as follows:

   Stock 1: Combine 51.2 µL Primary Atropine Stock + 1948.8 µL 1% normal serum - PBS (Final concentration 25.6 µg/mL).

   Stock 2: Combine 50 µL Stock 1 + 4950 µL 1% normal serum - PBS (Final concentration 256 ng/mL).

2. Dispose of the leftover Primary Atropine Stock as well as leftover atropine Stocks 1 and 2 at the conclusion of the RIA set up.

3. Prepare working dilutions of atropine standards as follows:

Tube #	Volume of Diluent[1]	Volume of Atropine Solution	Initial Atropine Concentration
1	3000 µL	1000 µL Stock 2[2]	64,000 pg/mL
2	2000 µL	2000 µL Tube 1	32,000 pg/mL
3	2000 µL	2000 µL Tube 2	16,000 pg/mL
4	2000 µL	2000 µL Tube 3	8,000 pg/mL
5	2000 µL	2000 µL Tube 4	4,000 pg/mL
6	2000 µL	2000 µL Tube 5	2,000 pg/mL
7	2000 µL	2000 µL Tube 6	1,000 pg/mL
8	2000 µL	2000 µL Tube 7	500 pg/mL
9	2000 µL	2000 µL Tube 8	250 pg/mL

[1]Diluent is 1% normal serum - PBS, pH 7.5
[2]Stock 2 = 256 ng/mL or 256,000 pg/mL

The volumes may be modified proportionately in order to produce the correct volumes for larger or smaller experiments. Dispose of the unused material at the end of the day.

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4. The RIA procedure is set up as described on the attached form entitled "Atropine Sulfate Radioimmunoassay Tube Setup." To use Program 38 on the TECAN model 5052, see Appendix A for the deck set up, Appendix B for the Assay Configuration, Appendix C for the Reagent Configuration, and Appendix D for the Pipetting matrix. Reagents are aliquoted to 12 x 75 mm polystyrene RIA tubes in order from left to right as indicated in the Atropine Sulfate Radioimmunoassay setup sheet.

5. Upon adding all reagents, vortex each tube 5-10 seconds.

6. Incubate the tubes 1 hour at 18-27°C.

7. Prepare the total counts tubes by adding 20 μL ^{3}H-atropine to each of the two RIA tubes, each containing 1.0 mL of sterile, distilled water.

8. After the RIA tubes have incubated for 1.0 hour, add 0.5 mL of 100 % Saturated Ammonium Sulfate to each RIA tube, excluding the total counts tubes. Vortex all tubes for 5-10 seconds. Incubate all tubes, including the total counts tubes, overnight at 4°C (approx. 18-24 hours).

Completion of RIA (Day 2)

1. Centrifuge RIA Assay tubes (excluding total counts tubes) at approximately 1550 x g for 30 minutes at 4°C. Carefully aspirate the supernatant with a transfer pipet and transfer the liquid to a radioactive waste container.

2. Add 1.0 mL 50 % Saturated Ammonium Sulfate to each assay tube (excluding total counts tubes). Vortex for 5-10 seconds. Centrifuge at approximately 1550 x g for 30 minutes at 4°C. Aspirate the supernatant with a transfer pipet and transfer the liquid to a radioactive waste container.

3. Add 1.0 mL sterile, distilled water (injection grade) to each tube (excluding total counts tubes) to dissolve the pellet. Vortex for 5-10 seconds.

4. Add 8.0 mL Hydrofluor Scintillation Fluid to each scintillation vial to be used.

5. Transfer the contents of each RIA tube, including the total counts tubes, to a separate scintillation vial by carefully pouring. Rinse all tubes with 2.0 mL Hydrofluor and transfer the fluid to the respective vial. Cap all scintillation vials and mix well.

6. Count the vials for 10 minutes or to a preset error of 2.0% on a liquid scintillation counter.

Data Analysis

1. Data analysis is performed using RiaCalc DM, Version 2.65 (Pharmacia Wallac). Data is reported as ng/mL.

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Critical Steps

1. See Appendices B-D for Program Application.

2. Use Program 38 on the TECAN RSP 5052. See Appendix A for the deck layout.

3. Use Injection grade, sterile distilled water to make PBS, pH 7.5, and the ammonium sulfate solutions, and to resuspend the final pellets.

   Foster Medical Supply, Cat. No. 2B0304

4. Use fine tip transfer pipets to aspirate the radioactive supernatant off the pellets.

   Baxter Scientific, Cat. No. P5214-13.

VI. QUALITY CONTROL

1. All equipment and instruments will be operated, calibrated, and maintained according to their respective SOPs.

2. The study director or his designee will review all raw data, completed data forms and other pertinent study records.

3. The form entitled "Atropine Sulfate Radioimmunoassay Tube Setup" details the contents of each standard, control, and sample tube and will be employed daily during assay set up to insure correct distribution of reagents.

4. The form entitled "Record For Instruments, Equipment, Reagents Used For Radio-immunoassay" will be used to document all reagents and equipment used in an assay.

5. The form entitled "Atropine Sulfate RIA Run List" will be utilized to record the identification and assay sequence for controls and samples for an assay.

6. Preparation of buffers and other reagents will be recorded on the attached form entitled "Buffer/Reagent Preparation."

7. A series of low, medium, and high controls are included in each experiment to assess the quality of each experiment. Control data will be tabulated for each run and will be reviewed by the study director.

8. Additional control parameters such as R/T, B/T, the slope and intercept of the regression curve and other parameters are computed by RiaCalc DM. These will be tabulated for each experiment and reviewed by the study director.

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BUFFER/REAGENT PREPARATION

Study:	
Project:	Date:
Buffer/Reagent:	
Buffer Storage Conditions:	Buffer Expir. Date:

Reagent	Supplier	Lot	Receipt Date	Expiration Date	Amount Used

Balance:	Description:	
	BCD ID:	Location:

Determination No.	Actual Wt.	Wt. Read
1		
2		
3		
4		

pH Adjustment (Reagent and volume): _____

pH Meter: BCD ID:_____ Final pH:_____

Comments:

_____

_____

Prepared by:_____ Date:_____

Reviewed by:_____ Date:_____

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ATROPINE SULFATE RADIOIMMUNOASSAY TUBE SETUP

STUDY CONTROL No.:	PROJECT No.:	
DATE:	RUN No.:	PAGE No.:

Tube No.	Conc.	Standard	Sample	Buffer	Normal Serum	Antibody	3H-Atropine Sulfates
Standard Curve							
1	T. Tube	None					20 µL
2	T. Tube	None					20 µL
3	NSB	None		330 µL	150 µL		20 µL
4	NSB	None		330 µL	150 µL		20 µL
5	0 pg	None		230 µL	150 µL	100 µL	20 µL
6	0 pg	None		230 µL	150 µL	100 µL	20 µL
7	0 pg	None		230 µL	150 µL	100 µL	20 µL
8	0 pg	None		230 µL	150 µL	100 µL	20 µL
9	3200 pg	50 µL Tube 1		180 µL	150 µL	100 µL	20 µL
10	3200 pg	50 µL Tube 1		180 µL	150 µL	100 µL	20 µL
11	1600 pg	50 µL Tube 2		180 µL	150 µL	100 µL	20 µL
12	1600 pg	50 µL Tube 2		180 µL	150 µL	100 µL	20 µL
13	800 pg	50 µL Tube 3		180 µL	150 µL	100 µL	20 µL
14	800 pg	50 µL Tube 3		180 µL	150 µL	100 µL	20 µL
15	400 pg	50 µL Tube 4		180 µL	150 µL	100 µL	20 µL
16	400 pg	50 µL Tube 4		180 µL	150 µL	100 µL	20 µL
17	200 pg	50 µL Tube 5		180 µL	150 µL	100 µL	20 µL
18	200 pg	50 µL Tube 5		180 µL	150 µL	100 µL	20 µL
19	100 pg	50 µL Tube 6		180 µL	150 µL	100 µL	20 µL
20	100 pg	50 µL Tube 6		180 µL	150 µL	100 µL	20 µL
21	50 pg	50 µL Tube 7		180 µL	150 µL	100 µL	20 µL
22	50 pg	50 µL Tube 7		180 µL	150 µL	100 µL	20 µL
23	25 pg	50 µL Tube 8		180 µL	150 µL	100 µL	20 µL
24	25 pg	50 µL Tube 8		180 µL	150 µL	100 µL	20 µL
25	12.5 pg	50 µL Tube 9		180 µL	150 µL	100 µL	20 µL
26	12.5 pg	50 µL Tube 9		180 µL	150 µL	100 µL	20 µL
Quality Control							
27	100 pg	50 µL Tube 6		180 µL	150 µL	100 µL	20 µL
28	100 pg	50 µL Tube 6		180 µL	150 µL	100 µL	20 µL
29	400 pg	50 µL Tube 4		180 µL	150 µL	100 µL	20 µL
30	400 pg	50 µL Tube 4		180 µL	150 µL	100 µL	20 µL
31	1600 pg	50 µL Tube 2		180 µL	150 µL	100 µL	20 µL
32	1600 pg	50 µL Tube 2		180 µL	150 µL	100 µL	20 µL
	Samples (Atropine Run List)		50 µL	180 µL	150 µL	100 µL	20 µL
	Samples (Atropine Run List)		50 µL	180 µL	150 µL	100 µL	20 µL

Performed By: _____ Date: _____

Reviewed By: _____ Date: _____

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ATROPINE SULFATE RIA RUN LIST
(Radioimmunoassay Laboratory)
Battelle, 505 King Avenue, Columbus, OH 43201

Date: _____ Run No.: _____ Page No.: _____

Study Control No.: _____ Project No.: _____

Tube No.	Sample ID or Source	Treatment	CPM	Average

Performed By: _____ Date: _____

Reviewed By: _____ Date: _____

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RECORD FOR INSTRUMENTS, EQUIPMENT, REAGENTS USED FOR RADIOIMMUNOASSAY

Project	Assay:	Project No.
		SC No.

LIST OF INSTRUMENTS/EQUIPMENT USED

SN	Instrument/Equipment	Model	Battelle ID	Location
1	Gamma Counter			
2	Scintillation Counter			
3	Water Bath (Temp.)			
4	Heating Blocks/Dry Bath (Temp)			
5	Incubator (Temp.)			
6	Refrigerator (Temp.)			
7	Freezer (Temp.)			
8				

LIST OF CHEMICALS, SOLVENTS, AND REAGENTS USED

SN	Name	Cat. #	Lot No.	Exp. Date
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

Comments:	
Performed By:	Date:
Reviewed By:	Date:

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STANDARDS FOR PROGRAM 38 ATROPINE RIA STANDARD CURVE: 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200 PG

Primary Atropine Stock at 1000 µg/mL

Diluent is 1% normal serum - PBS, pH 7.5

Dilution a: $\frac{51.2\ \mu L\ Stock}{1948.8\ \mu L\ diluent} = 25{,}600\ ng/mL$

Dilution b: $\frac{50\ \mu L\ Dilution\ a}{4950\ \mu L\ diluent} = \frac{1:10}{0} = 256\ ng/mL = 256{,}000\ pg/mL$ (12,800 pg tube)

Tube 1	1000 µL Dilution b / 3000 µL diluent	=	1:4	=	64,000 pg/mL	(3200 pg tube)
Tube 2	2000 µL Tube 1 / 2000 µL diluent	=	1:2	=	32,000 pg/mL	(1600 pg tube)
Tube 3	2000 µL Tube 2 / 2000 µL diluent	=	1:2	=	16,000 pg/mL	(800 pg tube)
Tube 4	2000 µL Tube 3 / 2000 µL diluent	=	1:2	=	8000 pg/mL	(400 pg tube)
Tube 5	2000 µL Tube 4 / 2000 µL diluent	=	1:2	=	4000 pg/mL	(200 pg tube)
Tube 6	2000 µL Tube 5 / 2000 µL diluent	=	1:2	=	2000 pg/mL	(100 pg tube)
Tube 7	2000 µL Tube 6 / 2000 µL diluent	=	1:2	=	1000 pg/mL	(50 pg tube)
Tube 8	2000 µL Tube 7 / 2000 µL diluent	=	1:2	=	500 pg/mL	(25 pg tube)
Tube 9	2000 µL Tube 8 / 2000 µL diluent	=	1:2	=	250 pg/mL	(12.5 pg tube)

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RIA CALCULATIONS SHEET

Date:_____ Page No._____

Study Control No._____ Project No._____

Total Counts (Average):_____ Background:_____

Entered By:_____ Reviewed By:_____

Tube No.	CPM	CPM-Background	% Bound	Average % Bound

BATTELLE COPY

RECORD OF INSTRUMENT/EQUIPMENT USAGE

Instrument/Equipment:_____

Battelle ID:_____

Entered By:_____ Date:_____

DATE	NAME	PROJECT	COMMENTS	*DAILY CHECK

* Daily Calibration Check, routine cleaning. Please initial this column.

Reviewed By:_____ Date:_____

BATTELLE COPY

SCHEDULED MAINTENANCE

EQUIPMENT:_____

MANUFACTURER:_____

BCD ID:_____/ SERIAL NO.:_____

REFERENCE SOP NO.:_____

DATE*	**PRESCRIBED MAINTENANCE	***INI.

*Prescribed maintenance will be completed +/- _____ week(s) of this date.

**Complete Record of Maintenance form upon completion of maintenance and enter completed form in instrument/equipment log book._____

Maintenance and cleaning of this equipment is the responsibility of _____, located in Room _____, phone_____. Contact this person in the event of equipment malfunction or failure.

***Please Initial and date this column to show completion of maintenance.

Maintenance scheduled by _____Date_____

Reviewed by_____Date_____

BATTELLE COPY

SCHEDULED CALIBRATION

EQUIPMENT:_____

MANUFACTURER:_____

BCD ID:_____/ SERIAL NO.:_____

REFERENCE SOP NO.:_____

DATE*	**PRESCRIBED CALIBRATION	***INI.

*Prescribed calibration will be completed +/- _____ week(s) of this date.

**Complete Record of Calibration form upon completion of calibration and enter completed form in instrument/equipment log book._____

Calibration and cleaning of this equipment is the responsibility of _____, located in Room _____, phone_____. Contact this person in the event of equipment malfunction or failure.

***Please Initial and date this column to show completion of maintenance.

Calibration scheduled by _____Date_____

Reviewed by_____Date_____

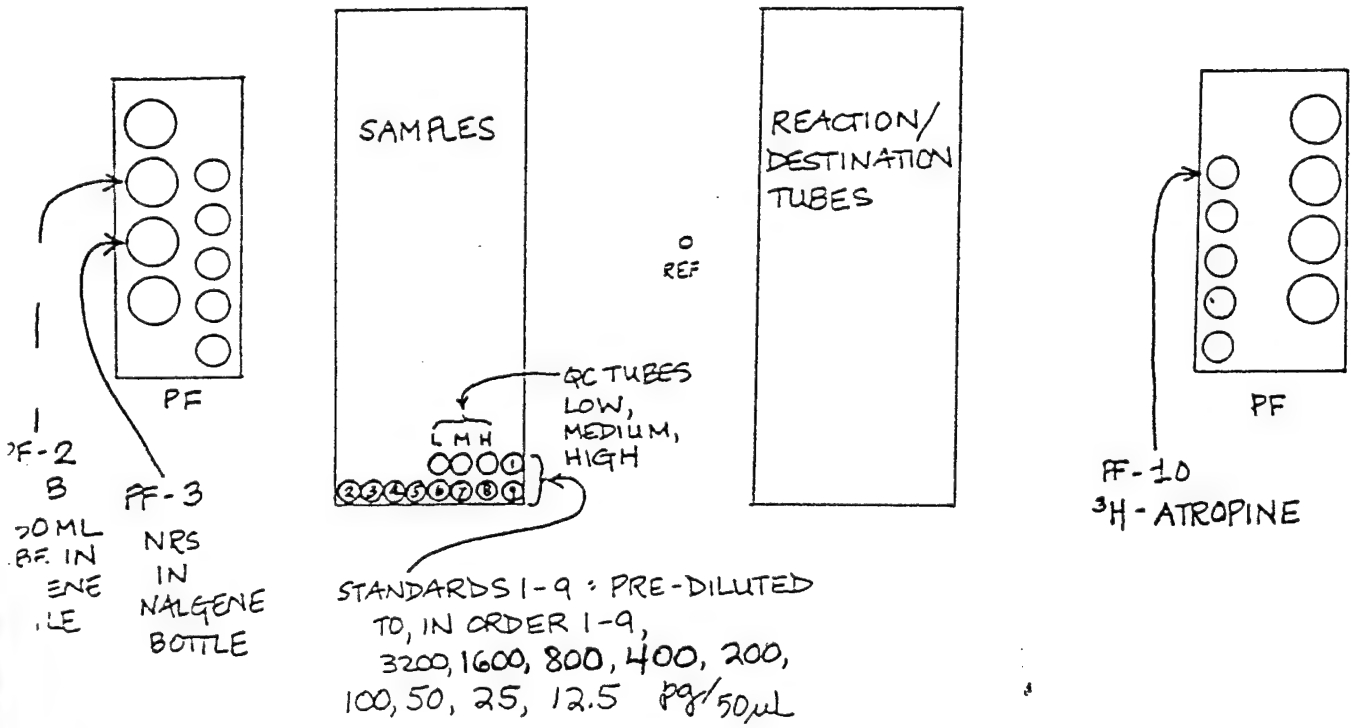
ATROPINE RIA (PROGRAM 38)

BATTELLE COPY

This program is identical to Program 8 except for the following changes: the volume of NRS delivered to each tube is changed from 50 uL to 150 uL for all tubes; the volume of standards delivered to each tube is constant at 50 uL; therefore, the standards must all be pre-diluted by hand.

NSB	=	330 uL PBS 150 uL NRS 20 uL ^{3}H-Atropine	Sample	=	180 uL PBS 150 uL NRS 100 uL Antibody 50 uL Sample 20 uL ^{3}H-Atropine
Standard Curve	=	180 uL PBS 150 uL NRS 100 uL Antibody 50 uL Standard 20 uL ^{3}H-Atropine			

DECK:



System Fluid: PBS Lines = PBS
DI H20 = DI H20

BATTELLE COPY

Method - 38 : Mod AtrRIA 50 uL Operator: Vic Moore Page - 2

R E A G E N T C O N F I G U R A T I O N

REAGENT-1: NORMSERA	When: Together with Samples
	How : Pipette Together with the Same Tip

Stirrer	Arm	Source Tray	Position in Tray
-	left	PF	3

REAGENT-2: ANTIBODY	When: Together with Samples
	How : Pipette Together with the Same Tip

Stirrer	Arm	Source Tray	Position in Tray
-	left	PF	2

REAGENT-3: 3H TRACE	When: Together with Samples
	How : Pipette with Other Arm, Wash betw. Tubes

Stirrer:	Arm	Tray	Source Pos.	Wash Volume	Waste Position	Flush Volume	Track:
-	right	PF	10	1000 µl	-	1000 µl	No

APPENDIX D

BATTELLE COPY

Method - 38 : Mod AtrRIA 50 uL Operator: Vic Moore Page - 3

P I P E T T I N G M A T R I X

Control Name	Tray	Pos	Vol. [µl]	Diluent [µl]	Wash [µl]	Rep-lica	Repeat #	1.Pos	NORMSERA [µl]	ANTIBODY [µl]	H TRACE [µl]
NSB	PF	3:		330	1500	2			150		20
ZERO	PF	3:		230	1500	4			150	100	20
STD 3200	TA	128:	50	180	1500	2			150	100	20
STD 1600	TA	129:	50	180	1500	2			150	100	20
STD 800	TA	130:	50	180	1500	2			150	100	20
STD 400	TA	131:	50	180	1500	2			150	100	20
STD 200	TA	132:	50	180	1500	2			150	100	20
STD 100	TA	133:	50	180	1500	2			150	100	20
STD 50	TA	134:	50	180	1500	2			150	100	20
STD 25	TA	135:	50	180	1500	2			150	100	20
STD 12.5	TA	136:	50	180	1500	2			150	100	20
Low Cont	TA	125:	50	180	1500	2	999		150	100	20
Low Cont	TA	125:	50	180	1500	2	30	10	150	100	20
Med Cont	TA	126:	50	180	1500	2	999		150	100	20
Med Cont	TA	126:	50	180	1500	2	30	20	150	100	20
High Con	TA	127:	50	180	1500	2	999		150	100	20
High Con	TA	127:	50	180	1500	2	30	30	150	100	20
SAMPLE		:	50	180	1500	2			150	100	20

APPENDIX C

FIGURES

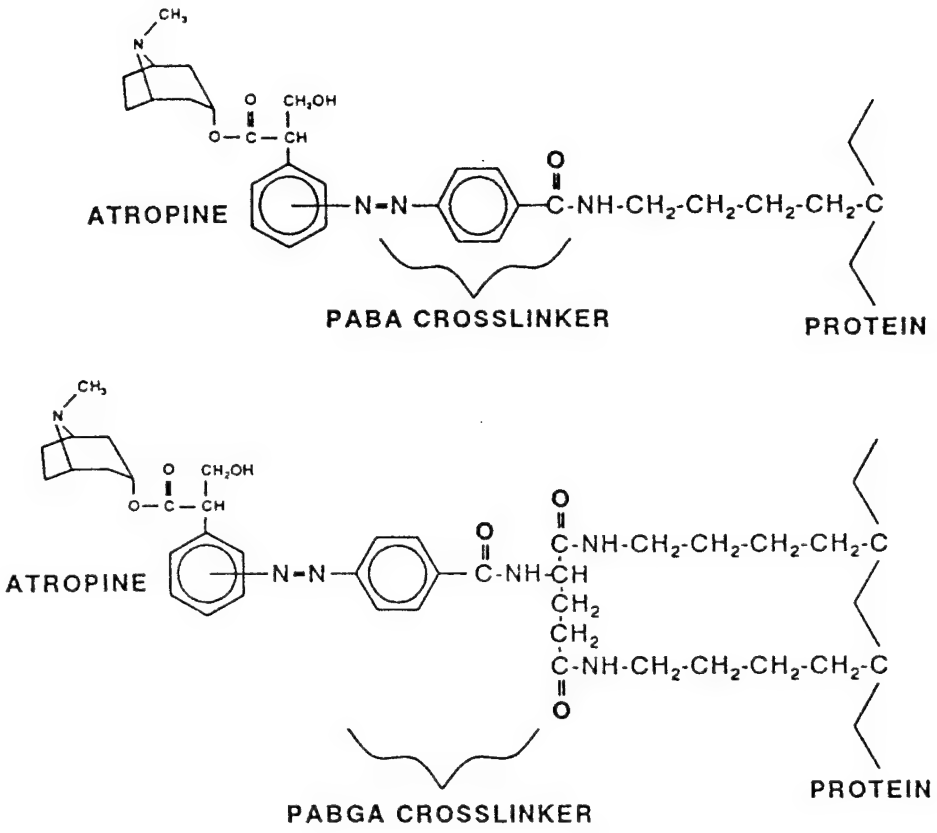


FIGURE 2. CONJUGATE CHEMISTRIES

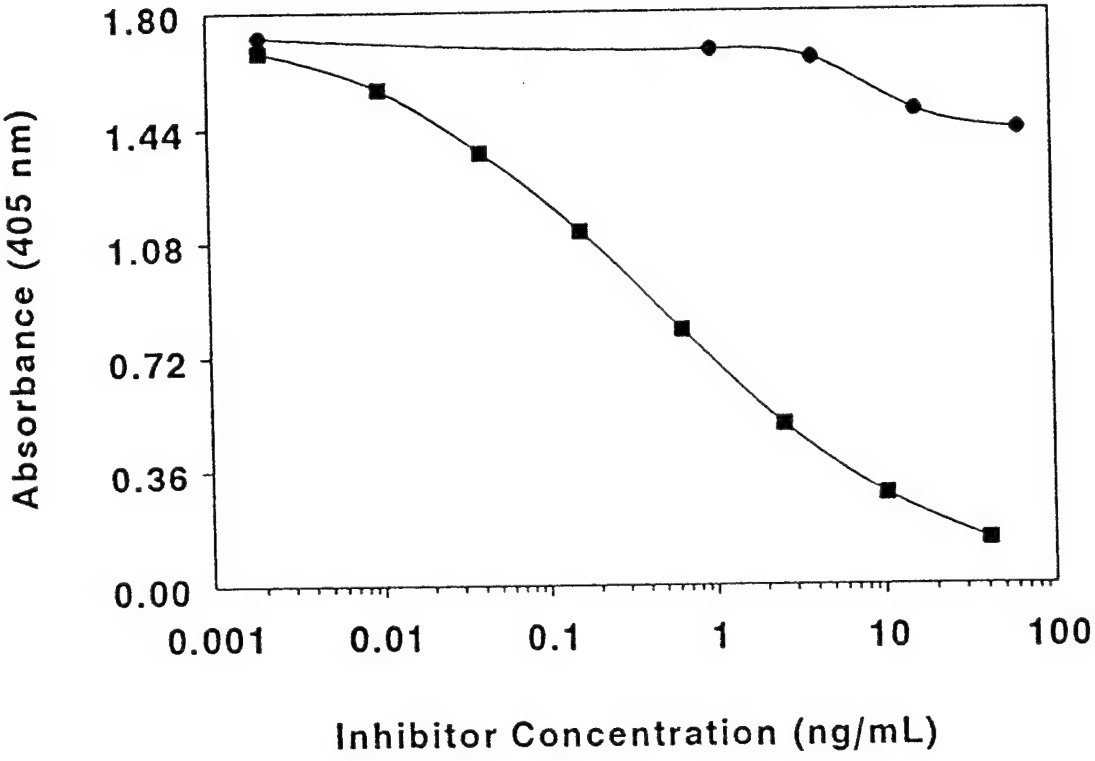


FIGURE 4. INHIBITION ELISA ANALYSIS OF ATROPINE-PABGA-TG

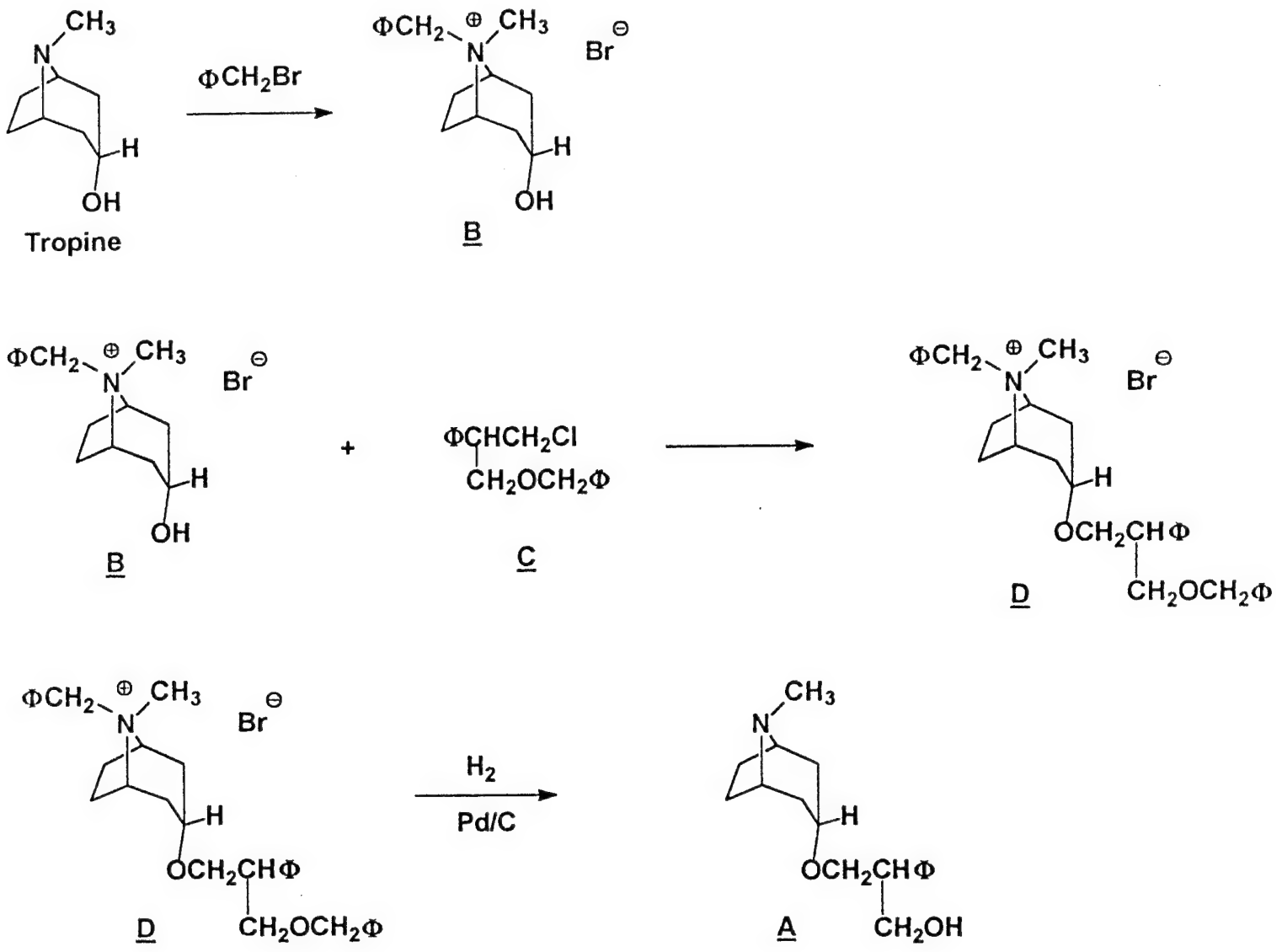


FIGURE 6. MULTIPLE STEP ROUTE TO ATROPINE ETHER DERIVATIVE (A)

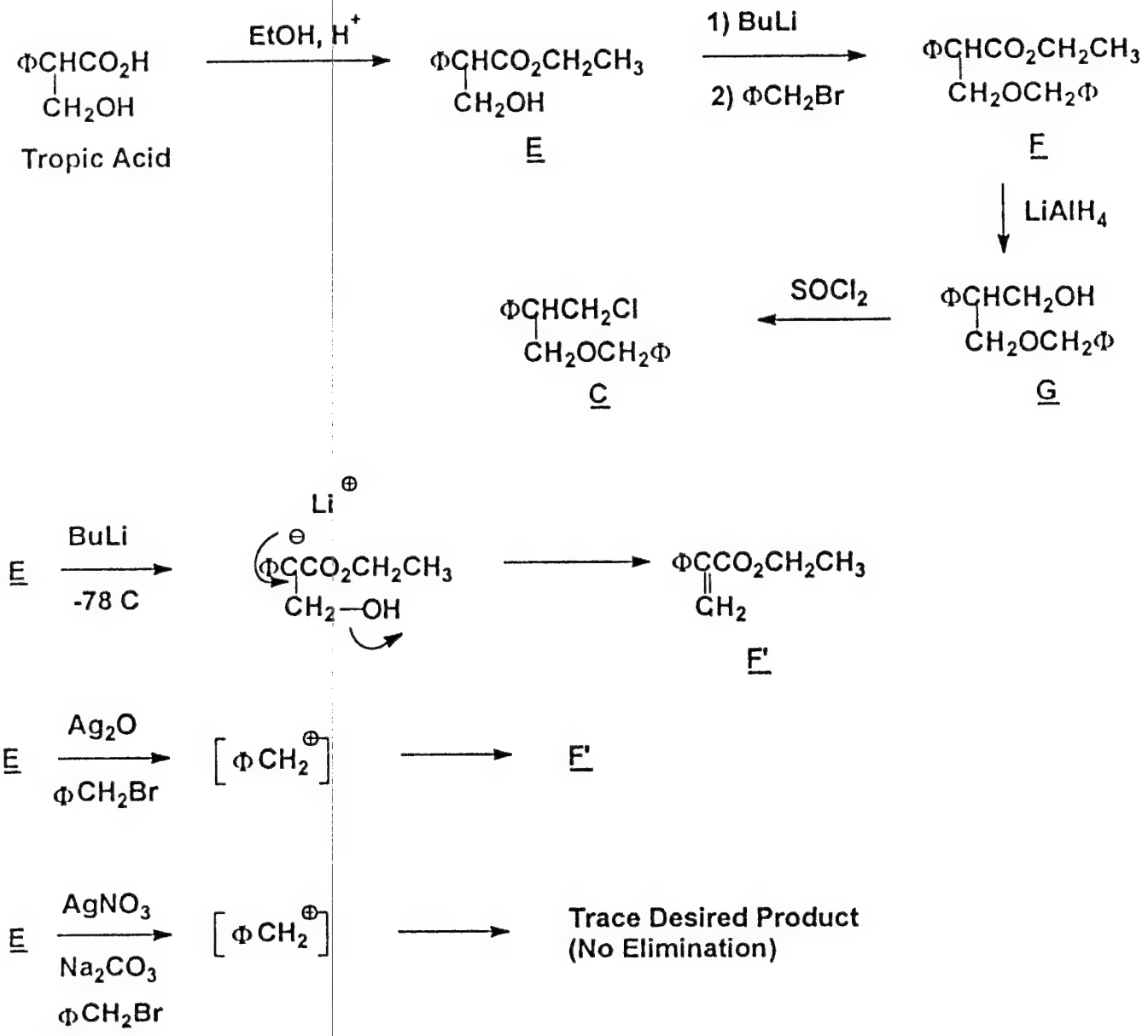


FIGURE 7. POTENTIAL ROUTE TO TROPIC ACID CHLORO/BENZYL ETHER DERIVATIVE (C)

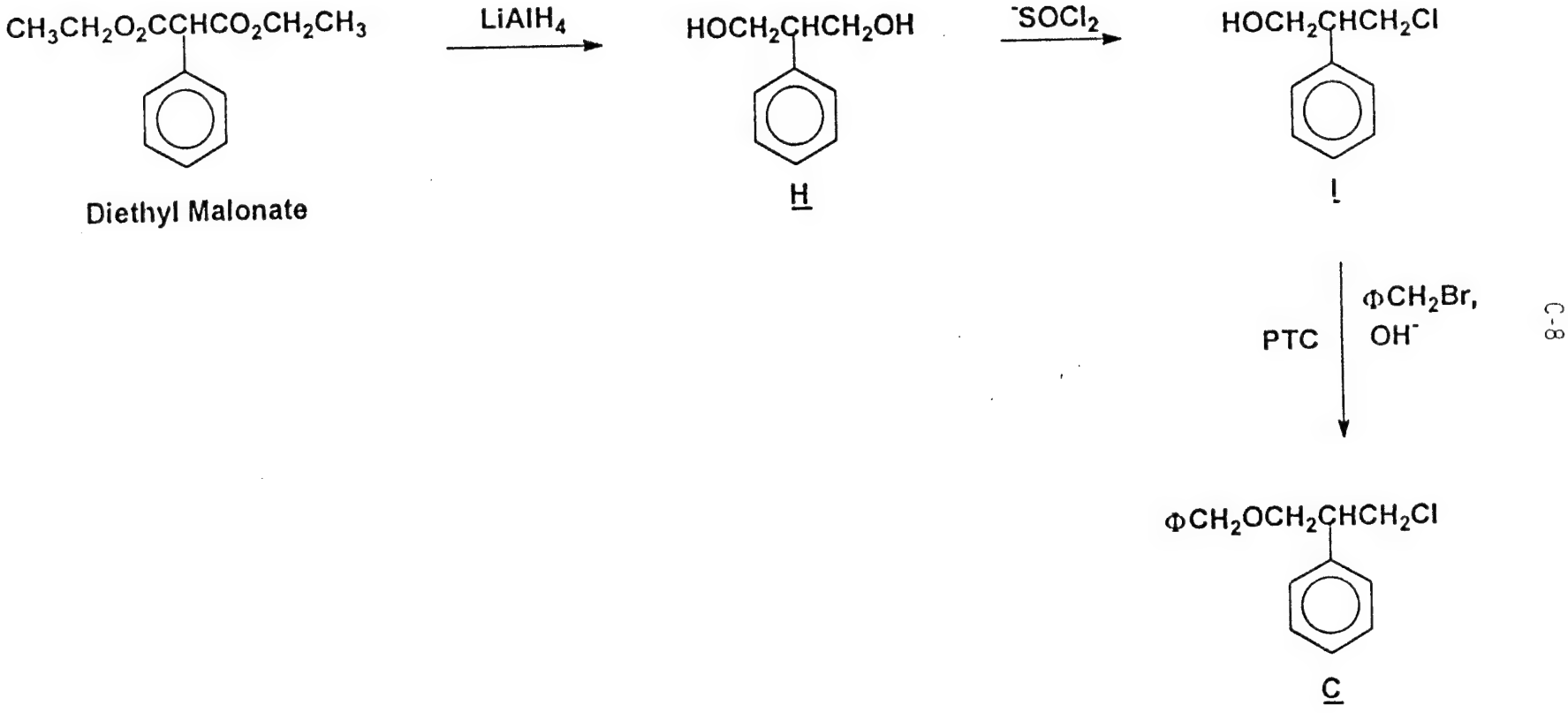


FIGURE 8. ALTERNATE ROUTE TO TROPIC ACID CHLORO/BENZYL ETHER DERIVATIVE (C)

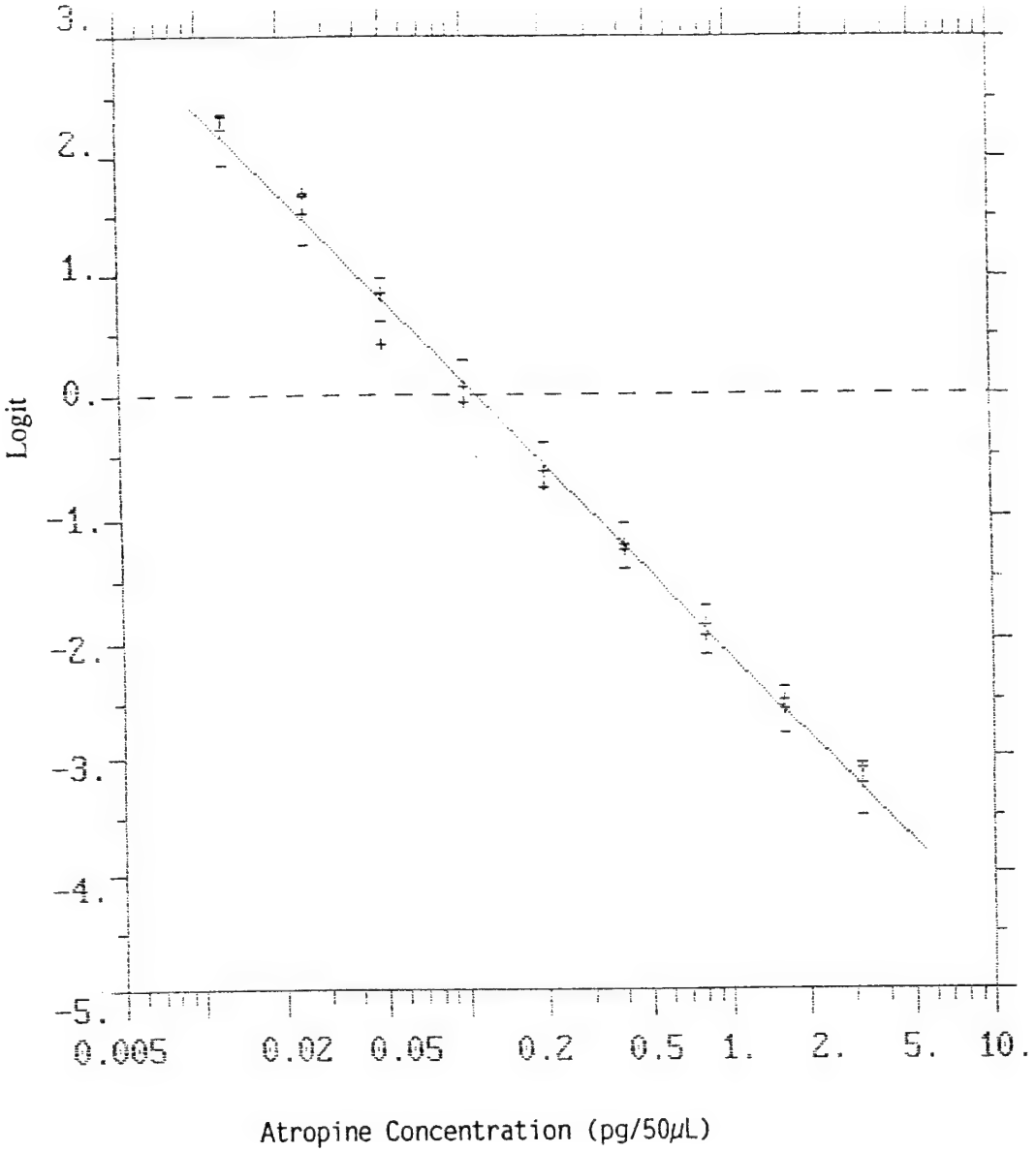


FIGURE 9. REPRESENTATIVE INHIBITION CURVE FOR IMPROVED RIA

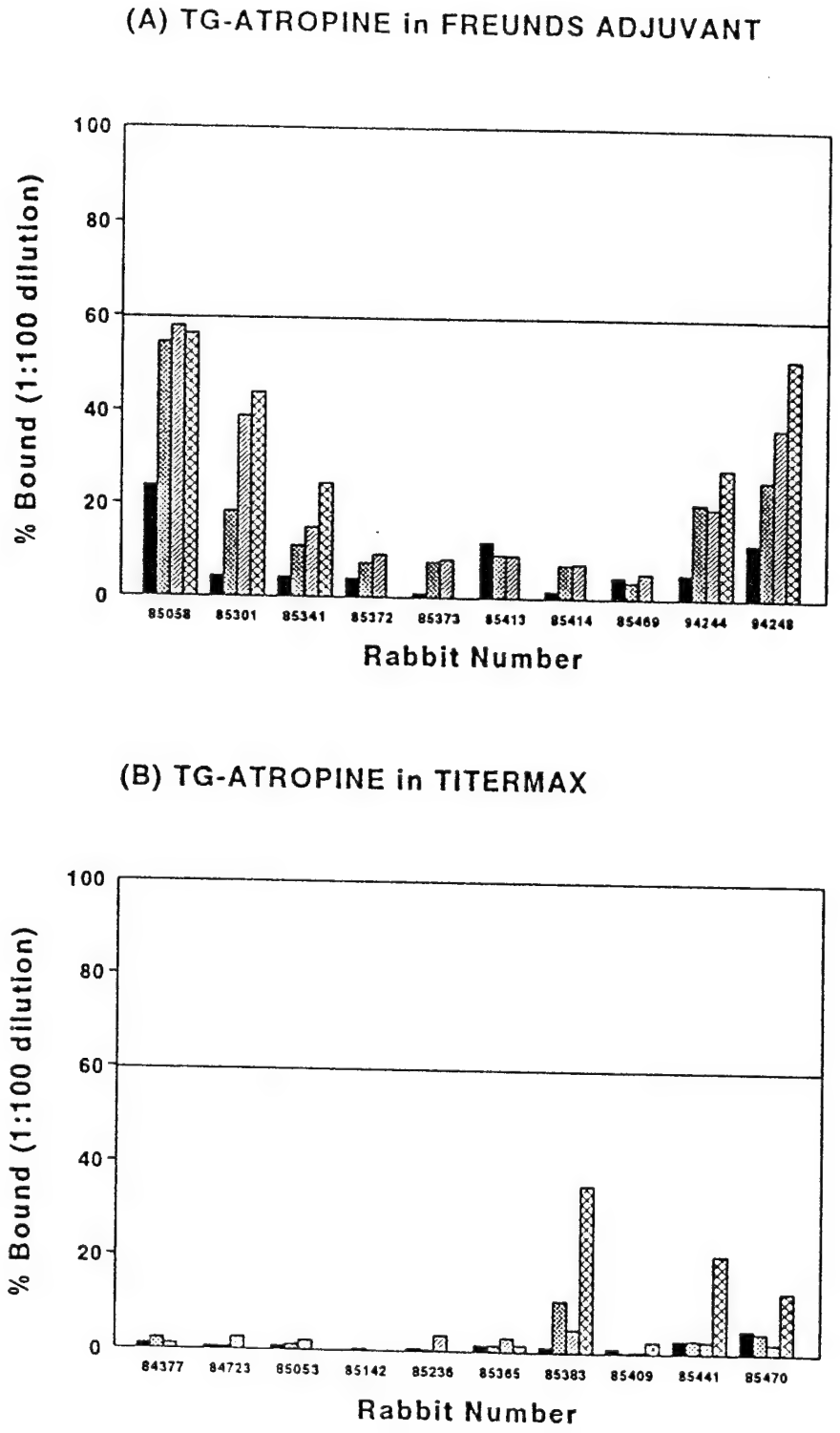
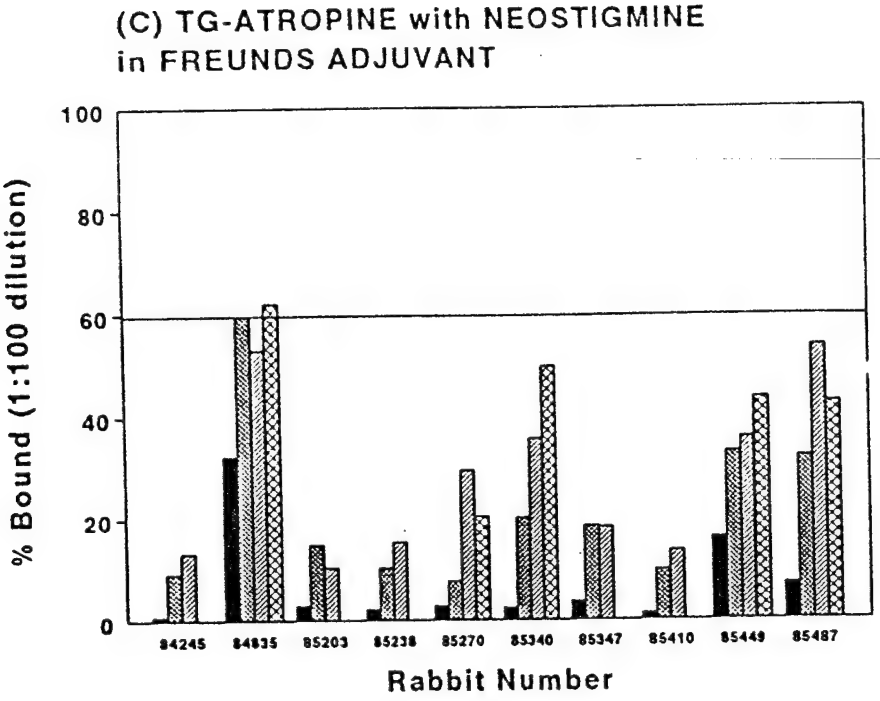


FIGURE 10. BINDING DATA FOR RABBIT ANTISERA

Bars represent data for each rabbit antisera on day 53 (solid), day 102 (small cross-hatch), day 144 (stripe), and day 214 (large cross-hatch).



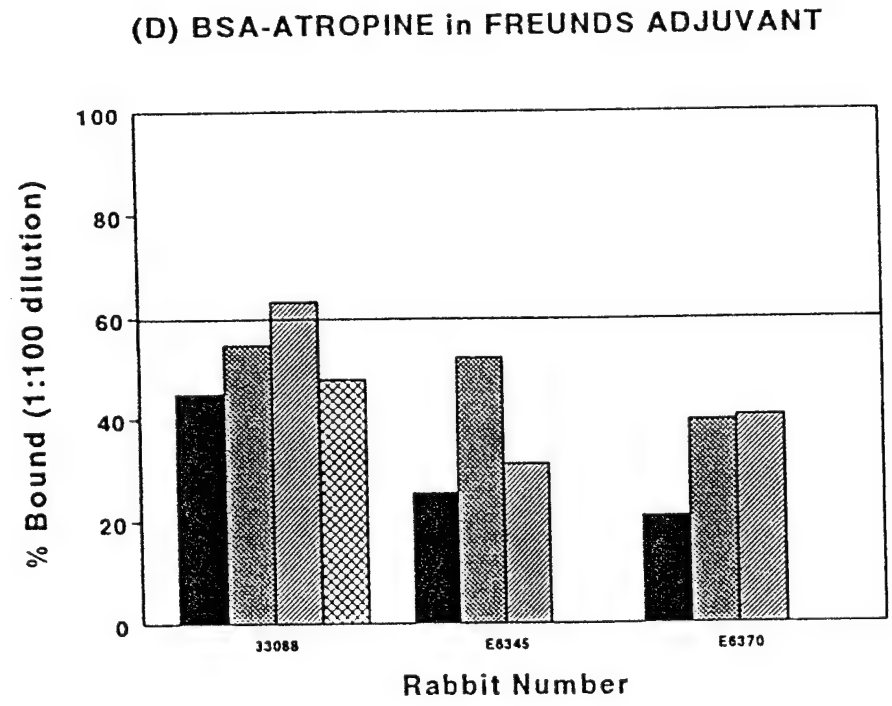


FIGURE 10. BINDING DATA FOR RABBIT ANTISERA (Continued)

Bars represent data for each rabbit antisera on day 53 (solid), day 102 (small cross-hatch), day 144 (stripe), and day 214 (large cross-hatch).

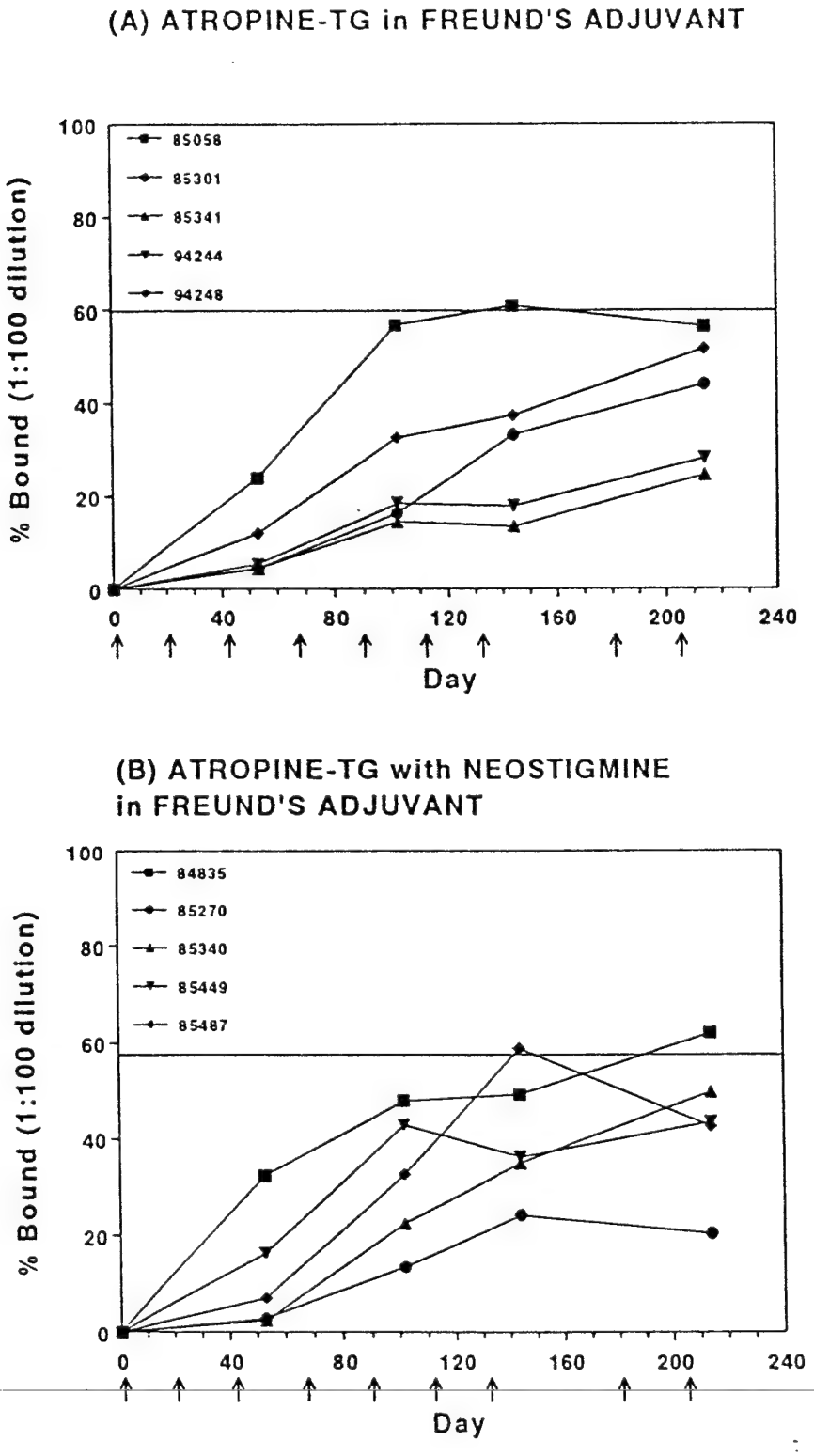


FIGURE 11. KINETICS OF ANTIBODY RESPONSE
Arrow indicates injection.

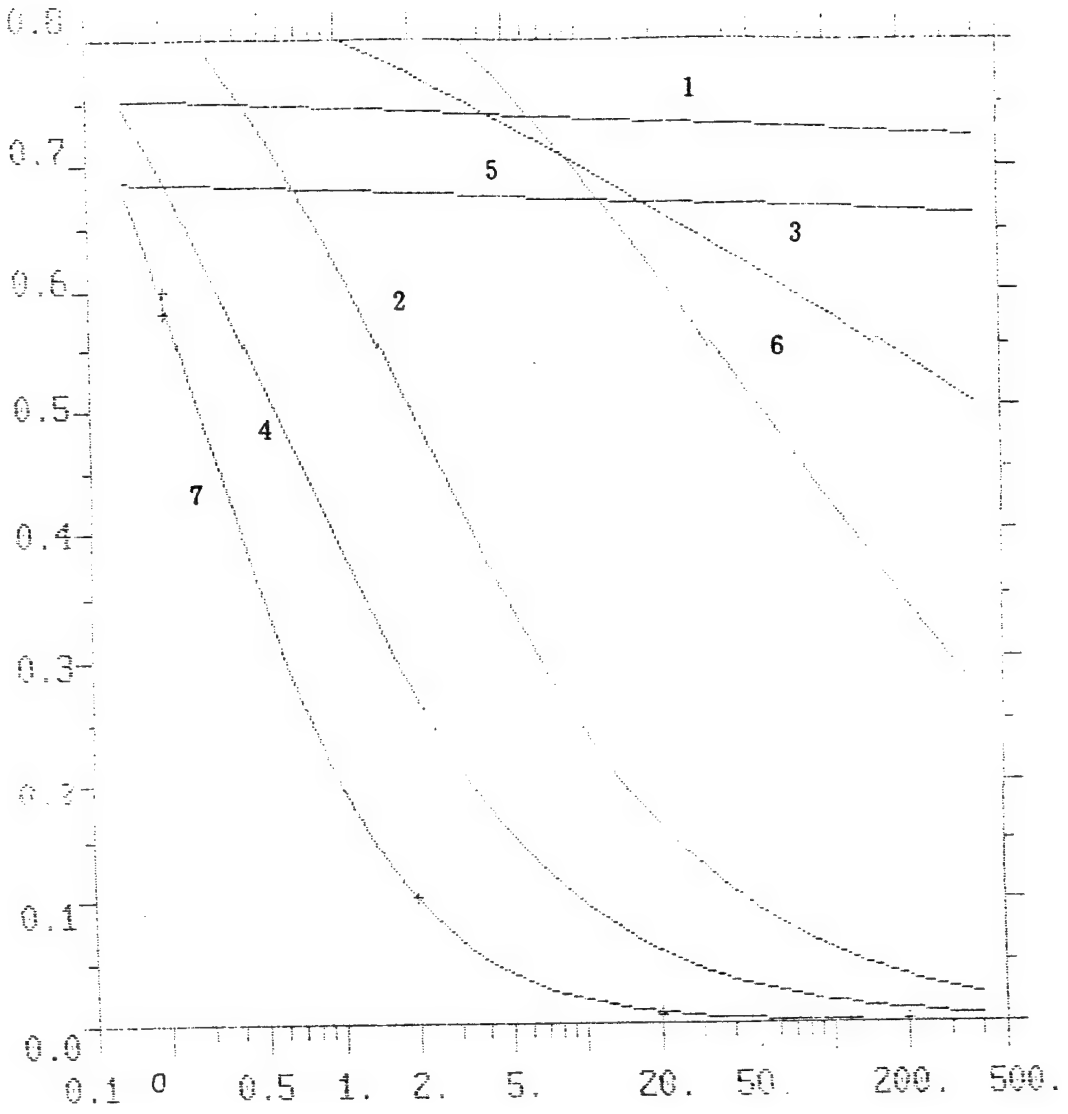
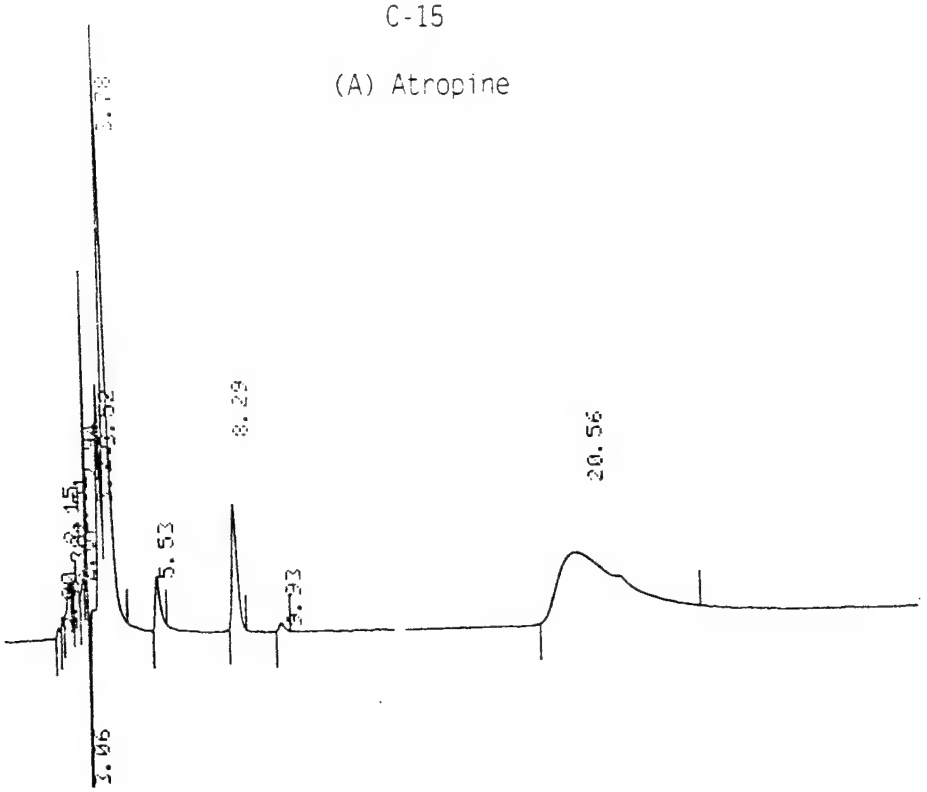


FIGURE 13. REPRESENTATIVE RIA SPECIFICITY STUDIES

Inhibition curves are shown for tropine (1), l-hyoscyamine (2), atropine methyl nitrate (3), d,l-homatropine (4), acetyl choline iodide (5), scopolamine (6), and atropine (7). Tropic acid is off scale.

(A) Atropine



(B) Tropic Acid

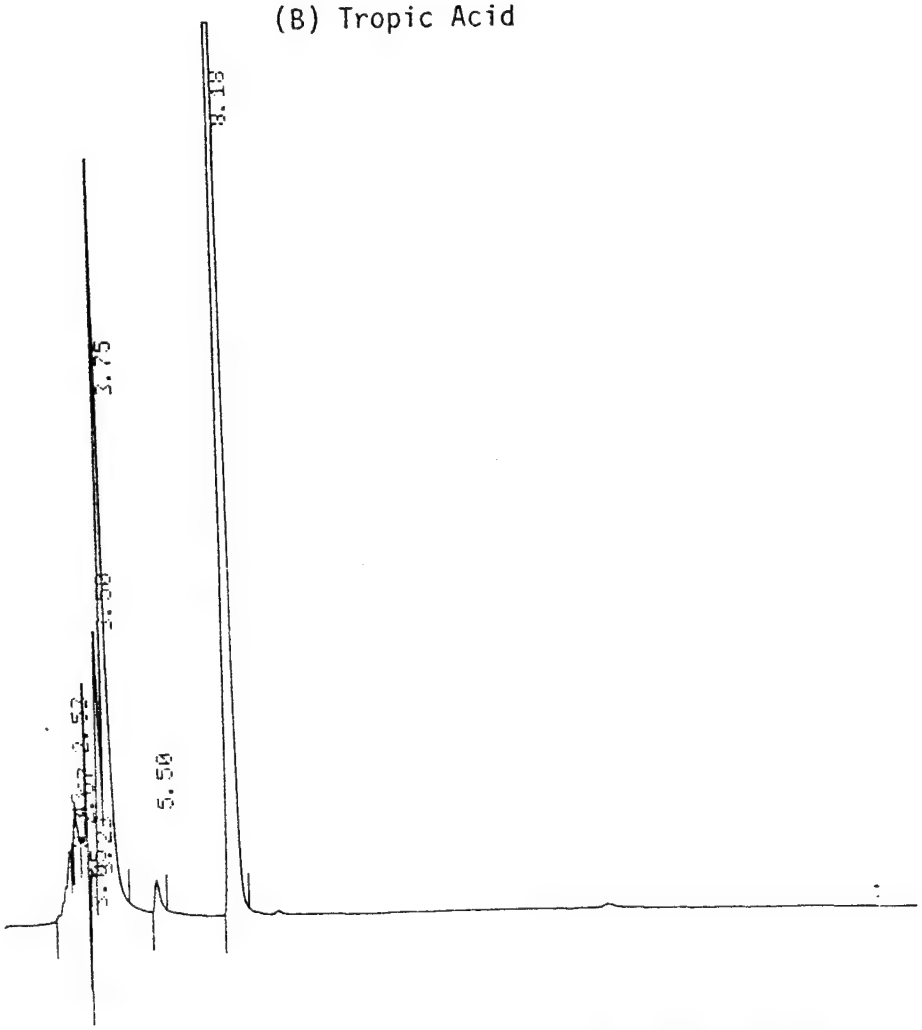
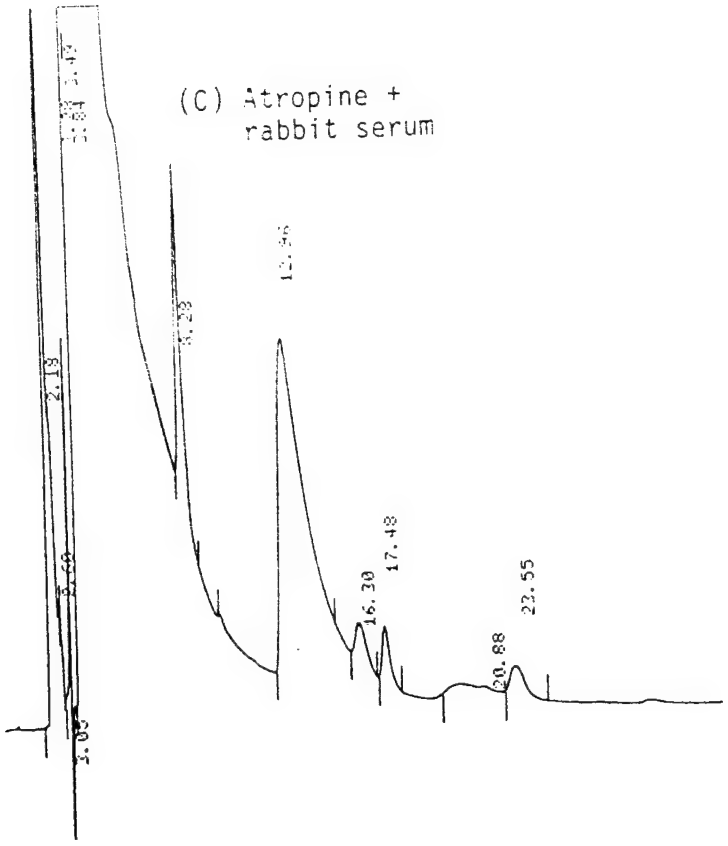


FIGURE 14. CHROMATOGRAMS FOR ESTERASE DEGRADATION STUDIES



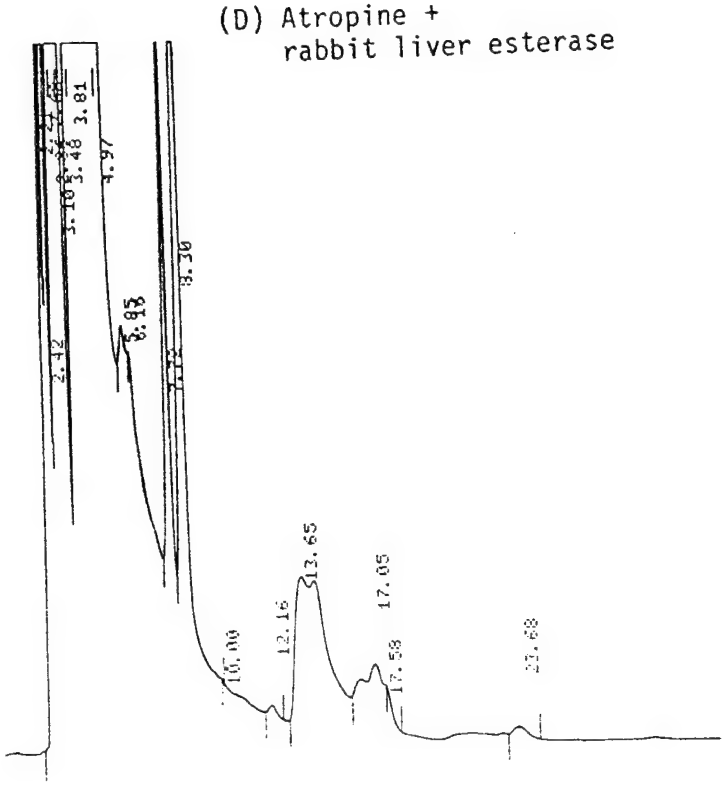


FIGURE 14. CHROMATOGRAMS FOR ESTERASE DEGRADATION STUDIES (Continued)

APPENDIX D

TABLES

TABLE 2. EVALUATION OF PARAMETERS FOR RIA IMPROVEMENTS

Parameter	Conditions Tested	Optimal Condition
tracer amount	4,000 cpm/20 µL PBS 10,000 cpm/20 µL PBS* 20,000 cpm/20 µL PBS 4,000 cpm/20 µL 1%NRS PBS	4,000 cpm/20 µL 1% NRS-PBS
normal rabbit serum	50 µL/tube* 150 µL/tube	150 µL/tube
anti-atropine dilution	1:100, 1:500, 1:600, 1:2000, and 1:2500 in 1% NRS-PBS	1:2000 in 1% NRS-PBS
standards	1000, 750, 500, 250, 100, 75, 50, 25, 0 in 10-50 µL PBS* 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5, 0 in 50 µL PBS 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5, 0 in 50 µL 1% NRS-PBS	3200, 1600, 800, 400, 200, 100, 50, 25, 12.5, 0 in 50 µL 1% NRS-PBS
PBS	1X* 10X diluted to 1X	1X
ammonium sulfate precipitation	RIA overnight at 4° C, then ppt for 1 hr at RT* RIA 1 hr at RT, then ppt overnight at 4° C	RIA 1 hr, then ppt
liquid scintillation counting time	count for 1 min* count for 10 min or to preset 2% CV	count for 10 min or to preset 2% CV

TABLE 3. IMMUNIZATION GROUPS

Group	Immunogen	Adjuvant	Number of Rabbits	Atropinase Status	Animal Numbers
1	atropine-PABGA-TG	Freund's	10	negative	85058 85372 85301 85373 85341 85413 94244 85414 94248 85469
2	atropine-PABGA-TG	TITERMAX	10	negative	85365 84377 85383 84723 85409 85053 85441 85142 85470 85236
3	atropine-PABGA-TG + neostigmine	Freund's	10	negative	84835 84245 85270 85203 85340 85238 85449 85347 85487 85410
4	atropine-PABA-BSA	Freund's	3	negative	33088 E6345 E6370
5	atropine-PABA-BSA	Freund's	10	unselected	1 2 3 4 5 6 7 8 9 10

TABLE 4. IMMUNIZATION SCHEDULE

Day	Date	Activity
0	10 Dec 92	serum sample 0 immunization 1
21	31 Dec 92	immunization 2
43	22 Jan 93	immunization 3
53	1 Feb 93	serum sample 1
68	16 Feb 93	immunization 4
90	10 Mar 93	immunization 5
102	22 Mar 93	serum sample 2
111	31 Mar 93	immunization 6
132	21 Apr 93	immunization 7
144	3 May 93	serum sample 3
181	9 Jun 93	immunization 8
202	30 Jun 93	immunization 9
214	12 Jul 93	serum sample 4
284	20 Sept 93	serum sample 5, bleed out

TABLE 5. ANTISERA TITER COMPARISON

Group	Immunogen	Adjuvant	Atropinase Status	Animal Number	Antisera Titer[a]
1	atropine-PABGA-TG	Freund's Adjuvant	negative	85058	800
				85301	800
				94248	400
				94244	100
				85413	100
				85373	<100
				85372	100
				85469	<100
				85414	<100
				85341	200
				Mean	280±275
2	atropine-PABGA-TG	TITERMAX	negative	85470	<100
				85441	<100
				85409	<100
				85383	<100
				85365	<100
				85236	<100

TABLE 5. ANTISERA TITER COMPARISON
(Continued)

Group	Immunogen	Adjuvant	Atropinase Status	Animal Number	Antisera Titer[a]
				85142	<100
				85053	<100
				84723	<100
				84377	<100
				Mean	<100
3	atropine-PABGA-TG + neostigmine	Freund's Adjuvant	negative	84835	>1600
				85340	400
				85449	800
				85487	1600
				85238	400
				85203	100
				84245	100
				85270	800
				85347	200
				85410	200
				Mean	620±546

TABLE 6. RIA PARAMETERS FOR ANTISERA

Parameter	Battelle[a]					WRAIR[b]
	84835	85487	85058	33088	9	
Initial Dilution	1:4000	1:1000	1:1000	1:4000	1:2000	1:1600
R/T	0.44	0.45	0.46	0.58	0.41	0.57
B/T	0.01	0.01	0.01	0.01	0.01	0.01
Slope	0.990	0.966	0.954	1.136	0.963	1.156
y-intercept	3.423	4.248	3.747	5.034	4.044	5.515
correlation coefficient	0.99985	0.99931	0.99962	0.99913	0.99931	0.99917
ED_{20} (pg/50 µL)	128.9	341.8	216.8	284.7	280.4	392.5
ED_{50} (pg/50 µL)	31.77	81.35	50.72	84.03	66.51	118.3
ED_{80} (pg/50 µL)	7.830	19.36	11.87	24.80	15.78	35.63
Detection Limit pg/50 µL ng/mL	12.5 0.25	12.5 0.25	12.5 0.25	12.5 0.25	12.5 0.25	75 1.5

TABLE 6. RIA PARAMETERS FOR ANTISERA
(Continued)

Parameter	Battelle[a]					WRAIR[b]
	84835	85487	85058	33088	9	
Response Range pg/50 μL ng/mL	12-3200 0.25-64	12-3200 0.25-64	12-3200 0.25-64	12-3200 0.25-64	12-3200 0.25-64	75-1000 1.5-20
Binding Constant	0.05 nM^{-1}	0.08 nM^{-1}	0.07 nM^{-1}	0.14 nM^{-1}	0.07 nM^{-1}	0.28 nM^{-1}

[a] Data based on a representative experiment inhibition curve (Figure 8) from sample analysis.

[b] Historical data.

TABLE 7. REPRESENTATIVE DATA FOR CALIBRATION POINTS CURVE

Calibration Point (Atropine, pg)	Rabbit Antisera					
	84835	85487	85058	33088	9	WRAIR
Total	4363.0[a] -[b] (1.1)[c]	4474.3 - (1.8)	4442.5 - (0.9)	4345.0 - (1.4)	4480.7 - (0.8)	nd[d] nd nd
NSB	45.2 - (8.6)	45.7 - (8.5)	47.5 - (9.5)	46.3 - (10.1)	43.0 - (1.5)	46.3 (7.8) -
0 pg	1926.2 43.6% (0.9)	2005.6 44.3% (1.8)	2037.8 45.3% (0.9)	2507.8 57.3% (2.8)	1855.1 40.8% (2.5)	2768.7 (1.0) 0[e]
12.5 pg	1407.8 31.6% (0.3)	1733.6 38.1% (1.0)	1616.6 35.7% (0.3)	2278.3 51.9% (1.4)	1545.8 33.9% (0.4)	nd nd nd
25 pg	1106.9 24.6% (0.6)	1534.5 33.6% (0.7)	1414.0 31.1% (1.7)	2034.0 46.3% (3.7)	1360.8 29.7% (1.8)	2399.1 (4.1) 25
50 pg	767.2 16.8% (0.3)	1270.5 27.7% (0.4)	1028.6 22.3% (1.1)	1619.2 36.6% (0.7)	1116.0 24.2% (7.9)	2017.8 (3.9) 50
100 pg	496.5 10.5% (0.5)	953.8 20.5% (1.3)	723.5 15.4% (0.8)	1104.2 24.6% (1.7)	772.7 16.5% (2.3)	1537.8 (1.8) 100

TABLE 7. REPRESENTATIVE DATA FOR CALIBRATION POINTS CURVE
(Continued)

Calibration Point (Atropine, pg)	Rabbit Antisera					
	84835	85487	85058	33088	9	WRAIR
200 pg	298.2 5.9% (3.1)	592.1 12.4% (3.9)	456.7 9.4% (3.4)	667.1 14.5% (0.2)	491.0 10.1% (2.5)	1150.6 (1.2) 150
400 pg	183.6 3.2% (2.7)	366.8 7.3% (2.6)	288.6 5.5% (0.7)	372.8 7.6% (1.7)	288.4 5.5% (4.3)	832.0 (5.1) 250
800 pg	117.8 1.7% (0.2)	231.4 4.2% (3.7)	178.4 3.0% (2.7)	216.1 4.0% (2.1)	192.6 3.4% (2.8)	462.5 (1.1) 500
1600 pg	83.9 0.9% (2.7)	148.3 2.4% (1.8)	117.8 1.6% (2.7)	135.6 2.1% (2.3)	124.3 1.8% (10.8)	344.8 (2.3) 750
3200 pg	65.4 0.5% (12.2)	107.4 1.4% (2.0)	87.1 0.9% (0.7)	89.8 1.1% (10.0)	88.9 1.1% (4.1)	271.8 (2.6) 1000

[a] counts per minute (cpm)

[b] $$\% \ Specific \ Bound = \frac{cpm_{test} - cpm_{bkg}}{cpm_{total} - cpm_{bkg}} \times 100$$

[c] %CV

[d] nd indicates no data

[e] pg/50 µL

TABLE 8. VOLUME OF ANTISERA

Rabbit	Bleed Date	Serum Volume	Total Volume	Titer	Total Tests[a]
84835	3 May 93	1.8 mL	22.3 mL	1:4000	446,000
	12 Jul 93	2.5 mL			
	20 Sept 93	18 mL			
85487	3 May 93	2.3 mL	27.8 mL	1:1000	139,000
	12 Jul 93	2.5 mL			
	20 Sept 93	23 mL			
85058	3 May 93	2.3 mL	28.3 mL	1:1000	141,500
	12 Jul 93	2.0 mL			
	20 Sept 93	24 mL			
33088	3 May 93	3.5 mL	41.8 mL	1:4000	836,000
	12 Jul 93	2.3 mL			
	20 Sept 93	36 mL			
Total			120.2 mL		1,562,500

[a] $$Total\ Tests = \frac{total\ mL\ antisera \times dilution\ factor}{0.2\ mL/test}$$

TABLE 9. SPECIFICITY OF ANTI-ATROPINE ANTISERA

Compound	Antisera					
	84835	85487	85058	33088	9	WRAIR
atropine sulfate	0.3[a] -	0.3 -	0.2 -	0.1 -	0.2 -	0.8 -
l-hyoscyamine	1.0 (30)[b]	2.0 (15)	0.6 (33)	0.2 (50)	1.6 (13)	33 (2)
d,l-homatropine	1.4 (21)	0.6 (50)	0.8 (25)	1.9 (5)	3.6 (6)	24 (3)
scopolamine	107 (0.3)	54.0 (0.6)	27.4 (0.7)	4.0 (3)	49.8 (0.4)	131 (0.6)
acetylcholine iodide	>200 (>0.2)	>200 (>0.2)	>200 (>0.1)	>200 (>0.05)	>200 (>0.1)	nd
atropine methyl nitrate	>200 (>0.2)	>200 (>0.2)	191 (0.1)	>200 (>0.05)	>200 (>0.1)	>500 (>0.16)
tropine	>200 (>0.2)	>200 (>0.2)	186 (0.1)	>200 (>0.05)	>200 (>0.1)	>500 (0.16)
tropic acid	>200 (>0.2)	>200 (>0.2)	>200 (>0.1)	>200 (>0.05)	>200 (>0.1)	>500 (>0.16)

[a] nM amount needed for 50% inhibition of atropine sulfate binding to antibodies.

[b] $$\% \ Crossreactivity = \frac{ID_{50} \ Atropine \ Sulfate}{ID_{50} \ Test \ Compound} \times 100$$

TABLE 10. RIA ANALYSIS OF ESTERASE DEGRADATION OF ATROPINE CONJUGATES

Treatment	Inhibitor		
	atropine	atropine-PABA-TG	atropine-PABGA-TG
untreated	0.164[a] (-)[b]	0.103 (-)	0.636 (-)
rabbit serum esterase	0.699 (23)	0.129 (80)	3.301 (19)
rabbit liver esterase	0.598 (27)	0.147 (70)	0.541 (118)

[a] nM needed for 50% inhibition of atropine sulfate to anti-atropine sera.

[b] $\% \ Binding = \frac{ID_{50} \ Untreated}{ID_{50} \ Treated} \ x \ 100$